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SECONDARY MALIGNANT DISEASE OF THE HEART.

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A study of the literature concerning malignant disease of the heart teaches that whilst primary tumours are rare, secondary deposits are not infrequently found. The following figures are illustrative. Scott and Garvin (1939), in a series of 11,100 autopsies, including 1,082 performed in cases of malignant disease, reported secondary malignant disease as follows—heart, 79 cases (7·3 per cent); pericardium, 61 cases (5·7 per cent); heart and pericardium, 140 cases (12·9 per cent). Willis reported an incidence of 6·2 per cent; Burke, 4·2 per cent; Helwig, 0·9 per cent; Pollia and Gogol, 2 per cent; Lymburner, 0·6 per cent; and Symmers, 1·6 per cent.

The subject is of more than academic interest, for with a better understanding it may be possible to establish a diagnosis during life in a greater number of patients, and the institution of certain forms of treatment, even though these are of a palliative nature. The symptomatology will therefore be reviewed, and attention drawn to the electrocardiographic findings in these patients.

This paper is based on a series of 51 cases of secondary malignant disease of the heart which were collected at the Royal Cancer Hospital from the autopsy records for the years 1930 to 1945 inclusive. The data obtained by analyses of these cases is set out in the following series of tables:

TABLE I.—*Secondary Malignancy of Heart. Sex Incidence.*

Total No.	Males.	Females.
51	23 (45·0 per cent).	28 (54·9 per cent).

TABLE II.—*Secondary Malignancy of Heart. Age Incidence.*

Years.	Under 20.	21-30.	31-40.	41-50.	51-60.	61-70.	71-80.
Number of patients	1	3	6	14	16	10	1
Percentage	1·9	5·8	11·7	27·4	31·3	19·6	1·9

TABLE III.—*Secondary Malignancy of Heart. Malignant Type and Region Involved.*

Malignant type.		Region involved.					
		Heart proper.		Pericardium.		Heart and pericardium.	
	No.	No.	%	No.	%	No.	%
Carcinoma	38	11	30·7	22	53·9	5	15·3
Sarcoma	10	4	40·0	5	50·0	1	10·0
Melanoma	3	3	100	0	0	0	0

TABLE IV.—*Secondary Malignancy of Heart. Primary Site and Region Involved.*

Primary site.	Region involved.		Combined.	Total No.
	Heart proper.	Pericardium.		
Breast	2	9	3	14
Stomach	1	3	0	4
Bronchus	0	4	0	4
Oesophagus	3	0	0	3
Uterus—body	1	1	0	2
Palate	1	0	0	1
Uterus—cervix	0	1	0	1
Parotid salivary gland	0	0	1	1
Kidney	1	0	0	1
Tonsil	0	1	0	1
Larynx	1	0	0	1
Tongue	1	1	0	2
Nose	1	0	0	1
Abdominal	1	0	0	1
Not identified	0	1	0	1
Lymphosarcoma	3	4	2	9
Hodgkin's sarcoma	0	1	0	1
Melanoma	3	0	0	3
Total number	19	26	6	51
Percentage	37·2	50·9	11·7	100

Pathological considerations.

In this series of cases there is a preponderance of females over males which may be accounted for by the relative large number of cases of carcinoma of the breast. The age incidence corresponds to that generally found in carcinoma, the maximum number of cases being found between the fifth and sixth decades.

Primary site.

The clinical records cited in the literature show that secondary malignant disease of the heart is associated with a primary tumour in almost every organ in the body, and this may be in an unexpected region. In illustration the following case is recorded: In 1905 a male patient aged 36 was admitted to the Royal

Cancer Hospital with an advanced carcinoma of the penis from which he died. At autopsy a metastasis was discovered in the form of a lobulated swelling measuring 1·5 × 1 cm., passing through the wall of the right auricle of the heart, and hanging into the chamber of the auricle as a tongue-like swelling measuring 4 × 2·5 cm., yellowish-white in colour. The pericardium contained a small quantity of clear fluid. Microscopic examination of the metastasis showed a squamous-cell carcinoma.

Reference to Table IV shows the large diversity of primary sites which give rise to secondary malignant disease of the heart, and reference is made to certain of these types in greater detail.

Carcinoma of the breast.—In this series of cases 27·4 per cent belong to this group. Secondary malignancy may manifest itself in the heart a long period after the primary disease is eliminated; thus in one patient radical mastectomy was performed 16 years previously, and in another 5 years before. The pericardium is usually involved and various types of disease are described. Focal collections of carcinoma cells may occur in the epicardial fat, the cells being transported along the dilated lymphatics. In other cases the pericardium is studded with nodules of growth, whilst a third variety consists of diffuse pericardial infiltration, and this process may extend into the pericardial fat. There may be commencing infiltration near the commencement of the great vessels. Another variety comprises flat plaques of growth embedded in the pericardium.

In a number of cases there is combined involvement of the pericardium and heart muscle. The growth in the pericardium may spread into the heart muscle by direct extension, or there may be a separate focus of carcinoma. Again, in one case, almost the whole of the heart muscle was infiltrated with nodules of growth.

Carcinoma of the stomach.—There are 4 cases in the series of this nature, the pericardium being involved in 3 and the heart muscle in 1. In the latter case a small nodule of growth was found in the wall of the left ventricle. When the pericardium is involved it may be thickened and diffusely infiltrated by tumour cells which can be seen filling the lumen of dilated lymphatics. In other cases there may be a localized nodule of growth, or a localized area of the pericardium is studded with small nodules.

Carcinoma of the bronchus.—There are 4 cases in the series and the pericardium is involved in all. The pericardium may be infiltrated by direct extension from the primary site, and in others the malignant cells are conveyed by the lymph channels. In the latter type of disease acute pericarditis may be present.

Carcinoma of the oesophagus.—Three cases are included in the series and the heart muscle was involved in all. In 1 case a deposit of carcinoma, 1·8 cm. in diameter, was present in the anterior aspect of the muscle of the right ventricle underneath the visceral pericardium. In another case, which had received radium treatment, a small irregular deposit of growth was present in the wall of the right auricle, the tumour cells showing keratinization. The third case showed massive invasion of the cardiac muscle, the growth extending deeply, and a few scattered groups of cells were seen in the fascicular planes.

Carcinoma of the uterus—body.—There are 2 cases of this nature in the series. In 1 case a metastasis was present in the heart muscle, with marked dilatation of the right auricle and ventricle. In the other case a deposit was found in the areolar tissue of the pericardium.

Carcinoma of the parotid salivary gland.—In this case there was a deposit of neoplasm in the subepicardial areolar tissue, from which spread had taken place to the appendix of the auricle and left ventricle through the lymphatics.

Malignant melanoma.—There are 3 cases in the series and the heart muscle was involved in all. In 2 cases it is known that the primary tumour was situated in the skin. The heart is usually involved extensively. In 1 case a subendocardial nodule was found in the right auricle, and on microscopy, in addition, a highly pigmented tumour was seen invading and destroying the muscle of the left ventricle; the cells of the tumour were very necrotic. Tumour cells were seen in the lumen of a small arteriole; obviously the disease in the heart was conveyed by the blood stream.

In another case nodules of growth were found in the muscle of the right auricle, left ventricle and left auricle; some of these were situated beneath the endocardium and growing into the auricle. The microscopic picture was similar to that of the primary skin tumour.

In the third case an amelanotic nodule was present in the upper and posterior part of the left ventricle, from which more extensive spread had occurred into the heart muscle.

Moragues (1939) reviewed the subject of cardiac metastases from malignant melanoma and found reports of 23 cases in the literature, to which he added 4 personal cases. In one of these cases there were signs of cardiac involvement; a loud systolic murmur was present in the pulmonary area, caused by a large tumour mass which almost occluded the pulmonary orifice.

The spread of malignancy to the heart.

This occurs by the usual four methods, namely, haematogenous, lymphatic, combined haematogenous and lymphatic, and by direct extension. In this series of cases there was definite evidence of all these modes. The relative frequency was worked out by Lymburner, who gave the following figures for a series of 36 cases of carcinoma and 16 cases of sarcoma, which he collected during the years 1915–1931:

TABLE V.—*Secondary Malignancy of Heart. Methods of Spread (Lymburner).*

Method of spread.	Carcinoma.	Sarcoma.
Haematogenous	72%	37%
Haematogenous and lymphatic	13%	12%
Direct extension	8%	50%
Lymphatic	5%	—

True haematogenous metastases are common in both carcinoma and sarcoma, the spread occurring along the coronary arteries. It may be that metastases are present already in the lungs, and it is probable that malignant cells invade the small pulmonary blood vessels and are carried by the pulmonary veins to the left heart, and from thence to the cardiac musculature along the coronary vessels, where numerous implants are produced. In cases with extensive lymphatic involvement, tumour cells may be carried along the lymphatic duct into the superior vena cava and thence to the heart. When the lymph nodes draining the pericardium and myocardium—the tracheo-bronchial nodes—are full of

malignant cells, retrograde spread back to the heart may occur. Direct extension from a bronchial or oesophageal carcinoma or a mediastinal neoplasm may involve the pericardium or myocardium.

Pathological states produced.

The presence of secondary malignancy of the *pericardium* produces various effects. If there is only an isolated nodule of growth present the rest of the pericardium is often normal. In other cases an effusion of straw-coloured fluid, varying in amount, is present; in some the effusion is blood-stained or purulent. The latter condition is seen with acute pericarditis due to malignancy. Sometimes the pericardial sac is obliterated by massive deposits of tumour, whilst in others there may be fibrous obliteration.

As a result of cardiac failure a hydrothorax may develop and ascites. In some cases a haemothorax is present. The heart becomes enlarged and it may alter in shape.

Symptomatology.

In all cases of malignant disease an accurate record of the patient's general physical condition is made when the patient initially comes under observation. In addition, a routine radiological examination is made of the chest, with special reference to the condition of the heart and lungs. Any subsequent abnormality which is noted can be compared with this initial investigation and inferences drawn.

In cases with tumours of the heart, Carnot and Lambling (1928) have described a clinical syndrome which resembles that of subacute bacterial endocarditis. Apart from this group of cases, attention is called to certain symptoms and signs which suggest cardiac involvement in patients known to be suffering from malignant diseases, especially of those organs which are recognized to give rise to secondary cardiac deposits.

Dyspnoea, tachycardia and cardiac irregularity are frequently outstanding symptoms, the irregularity being of the nature of either auricular fibrillation or auricular flutter. The symptoms may be correlated with the location of the tumour in the heart, causing heart block, or due to a pericardial or pleural effusion. It must not be assumed, however, that evidence of pericardial disease in a patient with malignancy is pathognomonic of involvement of the pericardium; acute pericarditis may occur in the advanced phase from other causes. Amongst important signs are those of cardiac enlargement and dysfunction, impaired resonance of the chest, the presence of abnormal breath sounds and added sounds. Cyanosis may be present and the signs of cardiac failure.

In the presence of this symptomatology certain investigations are carried out. When a pericardial effusion manifests itself, paracentesis is performed and cytological examination of the fluid is made. In a number of cases malignant tumour cells will be demonstrated. Radiological examination of the heart, including tomography, is carried out. Regarding the electrocardiographic findings, the case reports in the literature do not show uniformity; the results vary according to the position of the tumour in the heart. Siegel and Young (1933), dealing with this aspect of the subject, call attention to the work of Lloyd (1929), who published the first electrocardiogram in a proved case of tumour of

the heart. These tracings showed sino-auricular rhythm with a P–R interval up to 0·28 seconds—first degree A–V block. The tumour was situated in the region of the A–V node. Willius and Amberg (1930) published the electrocardiograph of a child who died with a cardiac metastasis from an endothelioma of the femur. This showed an incomplete bundle-branch block with regular T waves in leads 2 and 3, and a slight elevation of the S–T sequence in leads 1 and 2, with a depression of the S–T in lead 3. There was also a moderate degree of right axis deviation. Houck and Bennett in 1930 reported a polypoid fibroma occurring in the left auricle, and the electrocardiograph showed sinus tachycardia with a rate of 120. The ventricular complexes were abnormal and the electromotive force was low. The T waves in leads 1 and 2 were slightly diphasic. Digitalis had been given before the patient was admitted to hospital, which may account for the abnormality of the T waves. Siegel and Young (1933) reported the findings in a case of metastatic sarcoma of the myocardium. The T waves were inverted constantly in all leads, and there was no significant change daily. The S–T segments of all tracings were isoelectric or almost so. Hsiung, Szutu, Hsieh and Lieu (1940) describe the electrocardiographic findings in two patients with metastatic tumours in the heart which were diagnosed clinically. In one case of a reticulum-cell sarcoma of the nose with multiple metastases, including the heart and pericardium, the electrocardiograph showed evidence of coronary or myocardial disease. In the other case of lymphosarcoma, the electrocardiograph showed partial auriculo-ventricular block, prolonged conduction time, a tendency to right axis deviation and sino-auricular tachycardia.

Treatment.

It is not intended to discuss the general management of these patients, nor the treatment of cardiac failure when present. Attention is called to the possibility of irradiation in certain cases. When the primary tumour is known to be radiosensitive, high-voltage X-irradiation may prove of value in the diagnosis of metastases in the heart when enlargement is present. In these cases clinical and radiological evidence may be forthcoming of a marked reduction in the size of the heart and the disappearance of the pericardial effusion.

Shelburne and Aronson (1940) report the effects of high-voltage X-irradiation in a patient with metastases in the heart and pericardium from a primary myeloblastoma in the frontal bone. The treatment was given in an attempt to relieve the symptoms of a large pericardial effusion. In addition there was also complete heart block, indicating myocardial involvement. The effusion and heart-block disappeared and the patient was able to return to work for two months, but later died from the disease. A series of electrocardiographs showed the changes from complete heart-block to partial heart-block and normal rhythm.

SUMMARY.

The subject of secondary malignant disease of the heart is reviewed and a study presented of a series of 51 cases. Attention is called to certain pathological considerations. The symptomatology is described and the value of electrocardiography is stressed. The possible use of high-voltage X-irradiation in diagnosis and treatment is discussed.

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REFERENCES.

BURKE, E. M.—(1934) *Amer. J. Cancer*, **20**, 33.
CARNOT, P., AND LAMBLING, A.—(1928) *Bull. Mém. Soc. méd. Hôp. Paris*, **52**, 1773.
HELWIG, F. C.—(1935) *J. Kans. med. Soc.*, **36**, 265.
HOUCK, G. H., AND BENNETT, G. A.—(1930) *Amer. Heart J.*, **5**, 787.
HSIUNG, J. C., SZUTU, C., HSIEH, C. K., AND LIEU, V. T.—(1940) *Chin. med. J.*, **57**, 1.
LLOYD, P. C.—(1929) *Bull. Johns Hopk. Hosp.*, **44**, 150.
LYMBURNER, R. M.—(1934) *Canad. med. Ass. J.*, **30**, 368.
MORAGUES, V.—(1939) *Amer. Heart J.*, **18**, 579.
POLLIA, J. A., AND GOGOL, L. J.—(1936) *Amer. J. Cancer*, **27**, 329.
SCOTT, R. W., AND GARVIN, C. F.—(1939) *Amer. Heart J.*, **17**, 431.
SHELBURNE, S. A., AND ARONSON, H. S.—(1940) *Ann. Intern. Med.*, **14**, 728.
SIEGEL, M. L., AND YOUNG, A. M.—(1933) *Amer. Heart J.*, **8**, 682.
SYMMERS, D.—(1917) *Amer. J. med. Sci.*, **154**, 225.
WILLIS, R. A.—(1934) 'The Spread of Tumours in the Human Body.' London (Churchill).
WILLIUS, F. A., AND AMBERG, S.—(1930) *Med. Clin. N. Amer.*, **13**, 1307.

THE PROGNOSIS OF CARCINOMA OF THE BREAST IN RELATION TO THE TYPE OF OPERATION PERFORMED.

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SURGERY in the form of the so-called radical operation is in general at the present time the principal weapon in the treatment of carcinoma of the breast. What it can achieve and its limitations are now fairly well established. Thus it is known that if a patient with carcinoma of the breast has microscopical invasion of the axillary glands, the strong probabilities are that the disease has passed beyond the local area and that the patient will sooner or later succumb to the disease, although there will be a minority of fortunate exceptions. It is also known that if the axillary glands are not invaded the chances that the patient will be cured of the disease are appreciably increased. But one of the disappointing features of more recent figures is the diminution in the percentage of such cures. Thus at the time of Lane-Claypon's (1924, 1926, 1928) statistical analyses the figure of cures in the axillary gland free group was of the order of 70–90 per cent, while more recent figures in the main show a corresponding cure rate of 50–60 per cent or even less (Geschickter, 1943). There are probably two main

reasons for this. In the first place, it is now appreciated that there is no time after which the danger of recurrence may be said to have passed, and the more recent series of cases have tended to take this factor more into account in the form of longer follow-up times. Secondly there is a more critical attitude on the part of histologists to the diagnosis of carcinoma of the breast, and many cases of epithelial hyperplasia which fifteen to twenty years ago would unhesitatingly have been diagnosed as carcinoma would now no longer be. Some fifteen to twenty years ago also the question was raised whether irradiation should replace surgery in the treatment of carcinoma of the breast. But as the definitive treatment of the growth in the breast itself, irradiation has been largely abandoned except in cases with certain specific indications (e.g. acute carcinomata such as are typically met with during pregnancy or lactation, and cases in which there is well-marked skin involvement), because radiation in such a high proportion of cases fails to cause the complete disappearance of the primary growth.

The main desideratum in the treatment of carcinoma of the breast as in other forms of carcinoma remains the discovery of some general agent which will deal with deposits outside the field of local treatment. But until this ideal is attained we can only content ourselves with making the best use of the two local agents of proven value—surgery and irradiation. The present article attempts to assess the comparative results of a personal series of cases in which various techniques of treatment were used.

The technical surgical problem in carcinoma of the breast has been rendered clearer by modern work on lymphatic anatomy, and in particular by that of Gray (1939). This has shown, firstly, that the dermis is a plane rich in lymphatics and hence a rich potential plane of spread of carcinoma, particularly if the growth has spread into or near the skin ; and secondly, that the deep fascia is a plane devoid of or very poor in lymphatics, and hence not an important potential plane of spread. The former fact suggests greater radicalism in removal of skin, and the latter removes the main pathological reason for the routine sacrifice of the pectoralis major muscle. Accordingly, since the appearance of Gray's work we have modified our technique to remove more skin, in our more recent cases skin grafting in a high proportion, and we have gradually abandoned routine sacrifice of the pectoralis major, only removing it if actually invaded, which is rare and late. The argument is sometimes used that the removal of the pectoralis major is necessary for the adequate dissection of the axilla, and that in any case its nerve supply is bound to be cut. These technical arguments are not valid. With elevation of the arm, retraction of the pectoralis major and removal of the pectoralis minor it is easy to do a complete clearance of the axillary glands and fatty tissue right up to the apex of the axilla, and at the same time to preserve the lateral pectoral nerve, the main nerve supply to the pectoralis major. Apart from this modified radical operation there are smaller series of cases which have been treated in other ways, e.g. partial mastectomy combined with axillary dissection and simple mastectomy combined with irradiation to the axilla. At the end of 1946 we decided to look up and analyse our results. In all, the Middlesex Hospital cases treated under one of us (D. H. P.) in all ways from 1930–43 amounted to 118, and we followed them up until the end of 1946, i.e. 3–17 years. The figures, particularly when subdivided into the various groups, are small, but they can be reinforced by similar experiences in private practice.

COMPARATIVE RESULTS.

Standard radical and modified radical.

During the period under review 45 standard radical operations were performed and 46 modified radicals, the latter term being used for the operation above described, in which skin was removed more freely but the pectoralis major preserved. The comparison between the two types of operation is best made after each group has been subdivided into cases with and cases without histological invasion of the axillary glands. Of the cases with histological invasion of the axillary glands, 26 were operated by the standard radical operation, of whom 24 were traced. Of the 24 cases with glandular invasion submitted to a modified radical operation, 22 were traced. The comparative results of the two groups are given in Table I.

TABLE I.—*Cases With Axillary Glandular Involvement.*

	Standard radical.	Modified radical.
Total cases traced	24	22
Alive and well	2 (6 and 11 yrs.) 1 (alive and well 4 yrs. then lost trace.)	6 (3 to 7 yrs.)
Alive with disease	1 (11 yrs.)	1 (6 yrs.).
Died operation	1	1
Died other causes	1	1 (4 yrs.).
Died disease	18 (6 months to 7 yrs.)	13 (1 to 8 yrs.).

Of the cases without histological invasion of the axillary glands, 19 were operated on by the standard radical procedure, of which 18 were traced ; and 20 by the modified radical, of which 18 were traced. The comparative results are given in Table II.

TABLE II.—*Cases Without Axillary Glandular Involvement.*

	Standard radical.	Modified radical.
Total cases traced	18	18
Alive and well	8 (4 to 14yrs.)	13 (3 to 9 yrs.).
Died other causes	2 (7 and 8 yrs.)	0
Died disease	8 (1 to 10 yrs.)	5 (1 to 4 yrs.).

In addition, there were 2 cases of modified radical operations in which the condition of the axillary lymphatic glands was not noted histologically—1 died of the disease at 1 year, and 1 was alive and well at 8 years.

The results in both standard radical and modified radical are in accordance with the usual results in carcinoma of the breast in that only a minority of cases with glandular invasion live for long periods after operation, and that a considerably greater proportion so live if there has been no invasion of the axillary glands, although still a disturbingly high number die of the disease. The comparison between the figures of standard radical and the modified radical operation in this series is rendered more difficult in that the modified radical was evolved out of the standard radical, and hence has not been done for so long. The actual

figures for survival in both tables are better for the modified radical, but this is vitiated by the last-mentioned fact. If, however, comparative results are reduced to the lowest common denominator of the number of cases surviving for over 3 years, a time limit useless as an indicator of absolute but valid from the point of view of comparative prognosis, a fairer comparison is obtained.

TABLE III.—*Comparison of Three Year Survival after Radical and Modified Mastectomy.*

(a) *Cases with axillary involvement.*

	Standard radical.	Modified radical.
3 yr. survivals	11 out of 24 traced	10 out of 22 traced.

(b) *Cases without axillary involvement.*

	Standard radical.	Modified radical.
3 yr. survivals	14 out of 18 traced	15 out of 18 traced.

It would therefore seem fair to conclude that these figures bring no evidence to support the view that the addition of the removal of the pectoralis major muscle brings any increase in the survival figures, and to raise the question whether the routine removal of this muscle should remain an essential technical point.

A further way in which the two operations can be compared is by the comparative incidence of recurrence in the operative area, i.e. skin, chest wall and axilla. Out of 42 traced standard radical operations there were 10 cases with recurrence in the operative area, chiefly skin recurrences. Of these, 7 were cases with original lymphatic glandular involvement and 3 without such involvement. Out of 40 traced modified radical operations there were 7 local recurrences. Of these, 5 were cases with original lymphatic glandular involvement and 2 without such involvement. While the incidence in both is too high, and approximates to the 22 per cent noted by Truscott in the Middlesex Hospital series, the standard radical shows no lower incidence of local recurrence than the modified.

The 3 other types of procedure which have been carried out were :

(1) In the first place there were 10 cases in which partial mastectomy was combined with the usual full axillary dissection after the removal of the pectoralis minor.

(2) Secondly, there were 9 cases in which a simple removal of the breast was combined with irradiation to the axilla.

(3) Thirdly, there were 7 cases in which irradiation only of the breast was combined with axillary dissection.

The last group comprised cases which were all advanced, the glands being invaded in all. One case died as a result of the operation; the other 6 died of the disease at intervals varying from 6 months to 4 years after treatment. Moreover, in a high proportion of cases—4 out of 6 cases which survived operation—there was either failure of the irradiation to get rid of the original primary growth in the breast, or recurrence of the growth locally after apparent disappearance. In view of the advanced character of these cases, the ultimate fatal result was likely whatever form of treatment was adopted. But the high proportion of persistence or recurrence of the growth in the breast itself led to the abandon-

ment of this procedure. This experience confirmed that for the primary growth, at any rate, surgery is the simplest and most efficient treatment.

The 10 cases of partial mastectomy combined with dissection of the axilla were essentially early cases with small growths. Of the 2 cases with invaded glands, 1 was alive with the disease at 5 years and 1 died of other causes at 2 years. Of the 5 cases with glands not invaded, 3 were alive and well at 10 years, 9 years, and 5 years, 1 was alive with the disease at 8 years, and 1 had died of other causes 1 year after operation. There were also 3 cases in which no histological note was made of the condition of the axillary glands; of these, 1 was alive and well at 6 years and 2 died of the disease. From the point of view of survival after operation, this group compares favourably with other groups. But the procedure has been abandoned because of the occasional further development of carcinoma in the portion of the breast remaining. This happened in 2 of the cases of this series, and it also happened in other similar cases in a private series, in spite of the fact that, as already noted, only small and apparently early cases were considered suitable for this procedure, that a wide area of breast tissue around the growth was removed, and that post-operative irradiation was given as a routine. It was concluded, therefore, that while in an occasional case partial mastectomy combined with a dissection of the axilla might be justifiable—a conclusion supporting that of Fitzwilliams (1940) and of Mitchener, Bailey and Price (1937)—the danger of further development of carcinoma in the remaining breast tissue rendered the procedure an unwise routine.

The last group was of 9 cases in which simple removal of the breast was the only surgery performed, the axilla being treated by irradiation. These cases were usually elderly patients with apparently early growths. Of the 9 cases 7 were traced. Three were alive and well at 3 years, 4 years and 8 years after operation; 2 died of the disease, and 2 died of other causes. This procedure was abandoned, however, because it was found that the patients were more upset by the post-operative irradiation than were corresponding patients in whom the axilla was treated by surgical dissection.

Finally, there was one odd case of what appeared both clinically and histologically to be a scirrhus carcinoma of the breast in a girl aged 11 years which was treated by simple mastectomy. This patient in November, 1947 (15 years after operation), was alive, and well and about to be married.

X-rays.

Some of the patients after operation were treated by prophylactic irradiation and others were not. No consistent method of selection was adopted except that cases with glandular invasion were more likely to be treated. But there was no obvious difference in the results either in the gland invaded or the gland free group between those treated and those not. The absence of a regular method of selection, however, combined with the smallness of the figures renders this series unsuitable for any analysis of value on the question of the influence of prophylactic irradiation on the prognosis.

DISCUSSION.

The high incidence of bad results in treated cases of carcinoma of the breast has led to critical consideration of the standard treatment employed, namely radical operation. While there is fairly general agreement that in most cases

surgery, usually in the form of complete removal of the breast, is the simplest and most efficient way of getting rid of the primary growth, there is at the present time considerable diversity of opinion and practice on the question of what else should be done. Most surgeons still practise a standard radical operation. McWhirter (1947), following Keynes (1938), limits surgery to simple removal of the breast, treating the axilla entirely by X-rays. Handley and Thackray (1947), by demonstrating the frequency of internal mammary spread, have shown a gap in most methods of treatment of carcinoma of the breast, the best way of dealing with which is not yet settled. Our own approach has been a critical consideration of the detailed technique of the radical operation, and the results to date seem to show that routine removal of the pectoralis major in no way adds to the value of surgical treatment, thus affording some confirmation of modern views on lymphatic anatomy. The question might be rightly asked, what positive advantage is there in preserving the pectoralis major, granting that its routine removal is not necessary? To this we would reply that the main advantages of preserving the pectoralis major are three. Firstly, there is the cosmetic consideration, mutilation being much less with the pectoralis major preserved as compared with the skeleton-like prominence of ribs and costal cartilages after its removal. And if there is no curative advantage in removal of the muscle, cosmetic considerations, although secondary, are entitled to a place. Secondly, there is less loss of blood if the muscle is not removed, and consequently less operative shock. While in most cases this does not amount to a point of much practical importance, in old or feeble patients it may. With preservation of the pectoralis major combined surgical treatment of the breast and axillary contents is, in our experience, much less upsetting than simple mastectomy combined with irradiation to the axilla, and we have as already stated for this reason largely abandoned the latter procedure. And thirdly, the pectoralis major is a much more suitable bed for skin grafting than ribs and costal cartilages. And since, in view both of theoretical lymphatic anatomical considerations and of the general (Truscott, 1947) and our own personal experiences of the undue frequency of local skin recurrences, we now attach considerable importance to a wider removal of skin, the question of the suitability of the raw area left for skin grafting becomes of practical importance.

SUMMARY AND CONCLUSION.

1. The results are compared of different combinations of surgical and irradiation treatment of carcinoma of the breast in a personal series of 118 cases treated between 1930 and 1943, and followed up until the end of 1946.

2. Until an efficient general agent for the treatment of carcinoma of the breast is developed, a high proportion of cases are doomed to die of the disease whatever combination of local treatment by surgery and irradiation is used, because in such a high proportion of cases the disease has passed outside the field of local attack when the patient first comes for treatment.

3. A modified radical operation in which the pectoralis major is preserved shows results as good as those of the standard radical operation, and in addition has positive advantages.

4. Wider sacrifice of skin with subsequent skin grafting is desirable to reduce the incidence of local skin recurrences, which is still disconcertingly high.

5. Partial mastectomy combined with axillary dissection may be justifiable in occasional early cases with small growths, but is unjustifiable as a routine owing to the danger of local recurrence.

6. Simple mastectomy combined with irradiation to the axilla may give a satisfactory result, but the irradiation is generally more upsetting to the patient than surgical dissection of the axilla.

REFERENCES.

FITZWILLIAMS, D. C. L.—(1940) *Brit. med. J.*, **2**, 405.
GESCHICKTER, C. F.—(1943) 'Diseases of the Breast,' Philadelphia (Lippincott).
GRAY, J. H.—(1939) *Brit. J. Surg.*, **26**, 462.
HANDLEY, R. S., AND THACKRAY, A. C..—(1947) *Brit. J. Cancer*, **1**, 15.
KEYNES, G.—(1938) *Brit. med. J.*, **2**, 302.
LANE-CLAYPON, J. E.—*Rep. Minist. Hlth. Lond.*, No. 51, 1928; No. 34, 1926; No. 28, 1924.
MCWHIRTER, R.—(1947) *Lancet*, ii, 873.
MITCHINER, P. H., BAILEY, G. N., AND PRICE, A. K.—(1937) *St. Thom. Hosp. Rep.*, **2**, 192.
TRUSCOTT, B. MCN.—(1947) *Brit. J. Cancer*, **1**, 129.

THE EXCRETION OF 17-KESTOTEROIDS IN MEN OF DIFFERENT AGE-GROUPS, WITH SPECIAL REFERENCE TO PROSTATIC CANCER.

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ONE of the implications underlying the work of Huggins and Hodges (1941) on the factors governing prostatic hypertrophy is that malignant prostatic cells are not fully, if at all, autonomous. That is, they are still controlled in some measure by the same factors that control normal prostatic cells. Specifically, this is certainly true of their susceptibility to the influence of circulating androgens and oestrogens.

Carcinoma of the prostate is essentially a disease of old age. The possibility exists that the chief factor in deciding the individual's susceptibility to this disease may be his androgenic state, that is, the level of circulating androgens in his blood, particularly during the later decades of life.

The only practical method for ascertaining androgenic state is a determination of 17-ketosteroids excreted in the urine of the subject. In the hands of many different workers (Barnett, Henly, Morris and Warren, 1946) this method has revealed a very wide range of excretion of 17-ketosteroids by normal men, at least in the lower age-groups. An adult male may excrete in a range which is at least 7–28 mg./day. It is clear that if such differences persist throughout life

the prostates of individuals who excrete at the higher or lower end of this scale will be subjected to grossly different amounts of androgenic stimulation.

Most of the available data on the excretion of 17-ketosteroids is confined to results obtained in studies of men under the age of 30 years. Although there is a general impression that the excretion of these substances decreases with advancing age of the individual, there is a paucity of data on the range of excretion in the higher age-groups.

The ideal method of study would be to make repeated determinations of the 17-ketosteroid output in a large number of individuals over a period of many years, until, in fact, the subjects did or did not develop prostatic cancer. An alternative method, which has been adopted in the present work, is to compare the androgenic status, as judged by 17-ketosteroid estimations, of known cases of prostatic cancer with control values obtained on cancer-free subjects in the sufferer's own age-group. The experiments described in this paper were carried out for a two-fold purpose: Firstly, to obtain the control values necessary for comparison with those of prostatic cancer cases. Secondly, to discover whether the wide physiological range of excretion encountered in the lower age-groups persists in old age. The existence of such a range in old age might be a significant fact in any consideration of the aetiology of the disease.

METHODS.

The subjects used in this work, 128 in all, were either normal men or hospital patients suffering from diseases that do not affect the output of 17-ketosteroids. No cases of endocrine disease were included. Surgical cases were included only when the patients were in a late stage of convalescence and the effect of operative trauma on the excretion of 17-ketosteroids had ceased to exist.

Complete 24-hour collections of urine were made and aliquots hydrolysed and extracted according to the procedure of Robbie and Gibson (1943). The total 17-ketosteroids were estimated by the method described by Callow, Callow and Emmens (1938). The colorimetric measurements were made on a Hilger Spekker photoelectric absorptiometer using a green (Ilford Spectrum 604) and a violet (Ilford Spectrum 601) light filter. From the readings taken with these two filters a correction was made for the interfering chromogens in the urine extracts according to the method of Talbot, Berman and MacLachlan (1942). A parallel study of the validity of this colour correction which will be published elsewhere (Morris, Robinson and Warren, 1948) showed that such colour-corrected figures are a close approximation to the true 17-ketosteroid content. All the results given in the present paper have been so corrected.

RESULTS.

Although attention was mainly directed to males over the age of 40 years, since it is in this range of ages that chief interest lies from the point of view of prostatic cancer, some younger subjects were included in the present study. Fewer cases were investigated in this lower age-group, but the results obtained are in general agreement with those found by other workers (Barnet, Henly, Morris and Warren, 1946), and they are included in the summaries of all the results given in Table I and Fig. 1.

TABLE I.

Age group	0–5	6–10	11–15	16–20	21–30	31–40	41–50	51–60	61–70	71–80	81–90
Number of obs.	3	3	3	2	13	11	29	22	28	12	2
Mean 17-K.S. mg./day	0·7	5·8	7·9	12.6	14.1	16.8	11.0	7·7	7·8	6·4	4·3
σ	0·4	2·9	2·6	1·3	4·3	7·2	4·7	3·5	3·1	3·0	1·2
ε	0·2	1·7	1·5	0·9	1·2	2·2	0·9	0·8	0·6	0·9	0·9
Coeff. of var. per cent.	51	50	33	10	30	43	43	45	40	47	28
Range mean $\pm 2\sigma$	0–1·5	0–11·6	2·7–13·1	10–15·2	5·5–22·7	2·4–31·2	1·6–20·4	0·7–14·7	1·6–14·0	0·4–12·4	1·9–6·7

Summary of 17-ketosteroid excretions in normal males. σ = Standard deviation; ε = Standard error. Coefficient of variation (per cent.) is σ expressed as percentage of the mean value.

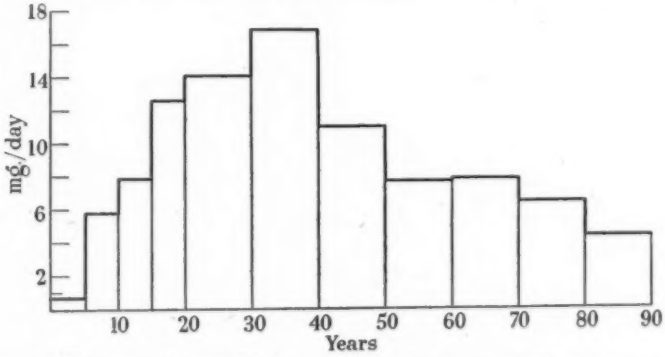

FIG. 1.—Mean excretion of 17-ketosteroids by normal men of different ages.

DISCUSSION.

Fig. 1 clearly shows the distribution of excretion of 17-ketosteroids with age. The output in childhood is low, rising rapidly at or about the age of puberty and reaching a maximum in middle age. The small difference in mean excretions between the third decade (14·1 mg./day) and the fourth decade (16·8 mg./day) is probably not significant ($P > 0{\cdot}05$), but the decreased excretion in the fifth decade (mean value 11·0 mg./day) is significant ($P < 0{\cdot}05$).

Consideration of the coefficients of variation brings out the interesting point that, although the general level of excretion decreases as age increases from 40 to 80 years, the scatter of individual excretions about the average value in any age-group remains constant. Expressed as a percentage of the mean output, men between 70 and 80 years of age show just as much variation in their excretions as men between 30 and 40. This is illustrated in Fig. 2, in which are plotted the mean excretions for the third to the eighth decade, together with the upper and lower limits of the ranges encountered at those ages. The ranges are computed as the mean values $\pm 2\sigma$.

The fact that men continue to show comparatively wide individual variations in their outputs of 17-ketosteroids and hence, probably, in the degree of androgenic stimulation to which their prostates are subjected, until very advanced age, must obviously be taken into account in any consideration of the aetiology of prostatic cancer. It will clearly be of importance to know whether known sufferers from this disease are characterized by excretions at the upper or lower ranges of their appropriate age-groups, or whether they are not significantly distinguishable

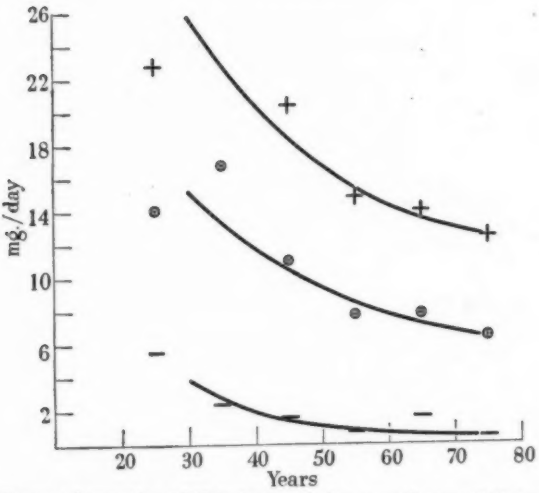

FIG. 2.—Range of excretion of 17-ketosteroids by normal men of different ages.
⊙ Mean excretion.
\+ Limit of upper range (mean + 2 σ).
— Limit of lower range (mean — 2 σ).

from the general population of those age-groups. An attempt to determine which of these alternatives is true is now in progress.

SUMMARY.

The excretion of 17-ketosteroids by normal men of all ages up to the ninth decade has been determined.

Excretion is low in childhood, rises rapidly at or about the age of puberty, and reaches a maximum in middle age. After the fourth decade there is a gradual decline in output.

From the fourth to the eighth decade the coefficient of variation of the excretion remains constant, and men between 70 and 80 years of age show just as great an individual variation in output as men between 30 and 40.

The implications of these results for the problem of prostatic cancer is discussed.

Generous grants to this Hospital by the British Empire Cancer Campaign have made this work possible. My thanks are also due to the Medical and Nursing Staff of St. Bartholomew's Hospital for much helpful co-operation.

REFERENCES.

BARNETT, J., HENLY, A. A., MORRIS, C. J. O. R., AND WARREN, F. L.—(1946) *Biochem. J.*, **40**, 778.
CALLOW, R. K., CALLOW, N. M., AND EMMENS, C. W.—(1938) *Ibid.*, **32**, 1313.
HUGGINS, C., AND HODGES, C. V.—(1941) *Cancer Res.*, **1**, 293.
MORRIS, C. J. O. R., ROBINSON, A. M., AND WARREN, F. L.—(1948) In preparation.
ROBBIE, W. A., AND GIBSON, R. B.—(1943) *J. clin. Endocrinol.*, **3**, 200.
TALBOT, N. B., BERMAN, R. A., AND MACLACHLAN, E. A.—(1942) *J. biol Chem.*, **143**, 211.

THE EFFECTS OF CHLOROETHYLAMINES ON TUMOURS, WITH SPECIAL REFERENCE TO BRONCHOGENIC CARCINOMA.

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DURING the war the pharmacology of the nitrogen mustards or chloroethylamines was studied intensively (Gilman and Philips, 1946). In the course of these investigations it was observed that tissues with a high proportion of dividing cells were particularly injured. This discovery led to the application of these substances in the treatment of malignant growth. The nitrogen mustards were injected into human patients suffering from cancers or leukaemia (Goodman, Wintrobe, Dameshek, Goodman, Gilman and McLennan, 1946; Wilkinson and Fletcher, 1947; Jacobsen, Spurr, Burton, Smith, Lushbaugh and Dick, 1946). There is no doubt that the procedure was justified, as many patients suffering from Hodgkin's disease must by now have benefited by treatment with these drugs (Rhoads, 1946). Experiments on tumour-bearing animals and trials on patients with bronchogenic carcinoma, using the following chloroethylamines are described.

(1) Methyl-*bis*-(β-chloroethyl)amine hydrochloride (HN2).
(2) *tris*(β-chloroethyl)amine hydrochloride (HN3).
(3) Dimethyl-β-chloroethylamine hydrochloride.

METHOD OF ACTION.

The vesicants, mustard gas and the nitrogen mustards, are toxic substances producing biological effects similar to those produced by ionizing radiations. Data available would suggest that a dose of 0·1 mg. per kg. of methyl-*bis*-(β-chloroethyl)amine is equivalent to a total body irradiation with about 50 r. The effects common to *bis*-(β-chloroethyl)amines and radiations include :

(*a*) Nausea and vomiting after moderate dosages.
(*b*) Haemoconcentration and death after 3 to 14 days following large doses.
(*c*) Thrombocytopenia leading to petechiae and extensive haemorrhages.
(*d*) Leucopenia.
(*e*) " Greying " of hair.
(*f*) Vesication.
(*g*) Disturbance of mitosis with " stickiness " and fragmentation of chromosomes, resulting in cell degeneration.
(*h*) Production of mutations in *Drosophila*, *Neurospora* and bacteria.

Treatment with polychloroethylamines (HN2 and HN3) forms a possible alternative to X rays for some diseases, particularly Hodgkin's disease. The

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effects seen in cases of bronchogenic carcinoma suggest that if the action of chloroethylamines could be increased (*a*) by modification of the molecule, (*b*) by improvement of the dosing schedule, or (*c*) by additional treatment with substances which might reduce the adverse effects, then useful results of definite clinical value might be obtained.

Treatment with chloroethylamines makes it possible to produce effects in tumours which are disseminated or localized in deep-seated tissues where radiation therapy is difficult. There have been indications that cases of Hodgkin's disease which have become resistant to X rays have responded to treatment with chloroethylamines.

ANIMAL EXPERIMENTS.

The animal experiments have indicated the importance of adequate dosage with substances of this type. If C3H mice with spontaneous mammary tumours or grafted lymphosarcomata, or rats with the Walker carcinoma, are given daily doses of 0·1 mg. per kg. of methyl-*bis*(β-chloroethyl)amine hydrochloride (HN2) by subcutaneous or intraperitoneal injection, negligible inhibition of tumour growth is obtained. With higher doses of the order of 1 mg. per kg. complete inhibition of the growth of the Walker rat carcinoma was obtained, and inhibition of spontaneous mammary tumours in mice occurred when doses of 0·4 mg. per kg. were given. Some experiments shown in Fig. 1 and 2 illustrate the increased effect of single massive doses over divided doses.

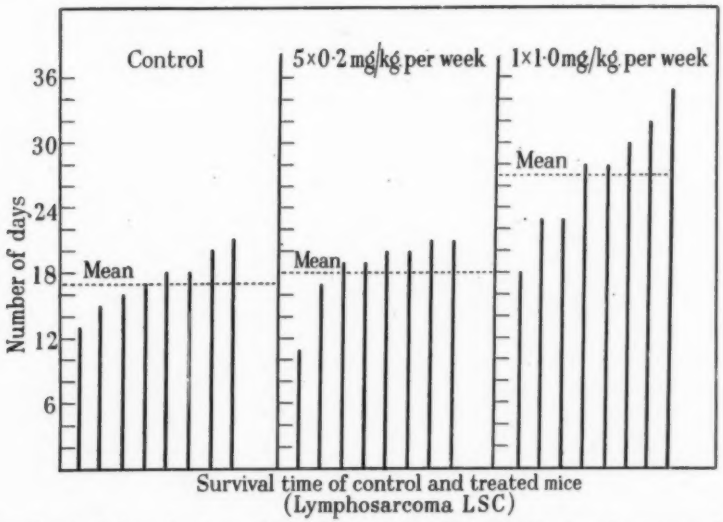


FIG. 1.—Survival times in days of C3H mice after grafting of the lymphosarcoma LSC. The first group of mice received no treatment, while the other groups were injected with methyl-*bis*-(β-chloroethyl)amine, HN2 with different schedules of dosing.

When the mice were given daily doses of *tris*(β-chloroethyl)amine hydrochloride (HN3) subcutaneously the treatment had very little effect on tumour

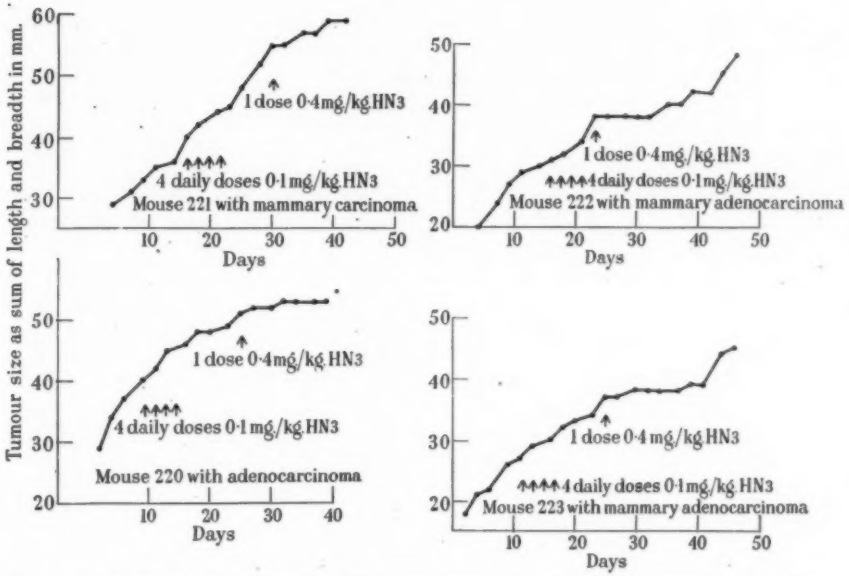

Tumour size as sum of length and breadth in mm.
1 dose 0·4 mg./kg. HN3
4 daily doses 0·1 mg./kg. HN3
Mouse 221 with mammary carcinoma
Days
1 dose 0·4 mg./kg. HN3
4 daily doses 0·1 mg./kg. HN3
Mouse 222 with mammary adenocarcinoma
Days
1 dose 0·4 mg./kg. HN3
4 daily doses 0·1 mg./kg. HN3
Mouse 220 with adenocarcinoma
Days
1 dose 0·4 mg./kg. HN3
4 daily doses 0·1 mg./kg. HN3
Mouse 223 with mammary adenocarcinoma
Days

FIG. 2.—The growth of spontaneous mammary tumours in mice given intraperitoneal doses of *tris*(β-chloroethyl)amine (HN3). The treatment was most effective when given in single large doses.

growth, but when later the same total amount was given in a single dose, inhibition of tumour growth occurred. Such inhibition was of temporary nature, lasting about one week in the case of the Walker rat carcinoma and up to two weeks in mice bearing spontaneous tumours. The effects of HN2, HN3 and dimethyl-β-chloroethylamine on spontaneous tumours are summarized in Table I. Rats bearing the Walker carcinoma have been given weekly subcutaneous injections of HN2—starting with 1 mg. per kg. for the first three weeks, and then reducing the dose to 0·5 mg./kg. at weekly intervals. A proportion (4/10) of rats treated in this way have been kept alive for three months, when all the control animals died within 21 days of grafting the tumour.

A spontaneous lymphosarcoma (L.S.C.) was also used. This tumour on transplantation into C3H mice usually kills the animals within 21 days, not by means of growth of the local tumour, but with extensive invasion of tissues. Treatment with small doses of HN2 had very little effect on survival of the mice. If treatment with weekly injections of 1 mg. per kg. HN2 was given commencing 10 days after implantation, the mice lived for from 18 to 35 days (Fig. 1).

Mice with spontaneous mammary tumours and mice with the grafted lymphosarcoma (L.S.C.) were treated with doses of 100 mg./kg. of dimethyl-β-chloroethylamine hydrochloride by subcutaneous injection. Such treatment when given repeatedly caused inhibition of some spontaneous mammary tumours (Table I), but had very little effect in prolonging the life of mice with transplanted lymphosarcoma. This compound given in single doses of 100 mg. per kg. to mice caused continuous tremor and incoordinated movement for several days.

TABLE I.—*The Effect of Treatment on Growth Rate of Spontaneous Mouse Tumours.*

In columns A, B, C and D the numerators in the fractions refer to the increase in size (length plus breadth in mm.) of the tumour, while the denominators refer to the number of days required for that growth : each column A, B, C and D refers to an individual mouse (Boyland, 1941).

Compound used in treatment.	LD 50, mg/kg.	Dose, mg/kg	(i) Growth in control period. (ii) Growth in treatment period. (iii) Ratio ii/i. A (i)	A (ii)	A (iii)	B (i)	B (ii)	B (iii)	C (i)	C (ii)	C (iii)	D (i)	D (ii)	D (iii)	Mean inhibition %.
Methyl-*bis*(β-chloroethyl)-amine hydrochloride (HN2)	4	4 × 0·1	16/9	17/14	0·68	13/14	3/14	0·23	10/14	9/14	0·90	8/14	7/14	0·87	33
	4	1 × 0·4	21/16	9/14	0·49	23/9	9/14	0·25	15/12	4/14	0·23	15/16	−3/14	−0·23	81
Tris(β-chloroethyl)amine hydrochloride (HN3)	2	4 × 0·1	18/12	9/14	0·86	11/12	15/14	1·17	9/12	9/7	1·33	9/9	10/14	0·72	−2
	2	1 × 0·4	9/14	2/14	0·22	15/14	4/12	0·31	9/7	2/14	0·11	10/14	2/14	0·20	79
Dimethyl-β-chloroethyl-amine	400	4 × 100	22/9	5/14	0·29	19/14	−1/14	0·05	10/11	16/14	1·26	7/14	5/14	0·71	45

Neurotoxic effects were never seen with mice dosed with HN2 or HN3 in doses up to 1 mg. per kg., although larger doses of both substances which have been hydrolysed in neutral solution will cause rapid death with convulsions (Boyland, 1946).

An interesting effect noticed in mice was the bleaching of hair at the site of injection (Fig. 3). It seems that subcutaneous injection of HN2 inhibits pigment formation in the skin of black or brown mice at the site of injection without having any effect on the growth of hair. The effect is similar to that induced by radioactive substances ; it is identical with the phenomenon of depigmentation in mice caused by subcutaneous implantation of plutonium (Brues, 1947, personal communication). Thus " bleaching of hair-colour " is another example of the radiomimetic actions of the *bis*-chloroethylamines.

The metabolism of Walker carcinoma tissue from rats dosed with 1 mg. per kg. HN2 was studied using the Warburg technique. A reduced glycolysis was found for six days following a single treatment (Fig. 7), with no change in the tumour respiration. The nature of the inhibition of glycolysis has not been examined further, but it is probably due to inhibition of hexokinase or other phosphate-transferring enzymes concerned in glycolysis. Dixon and Needham (1946) found that vesicants—either mustard gas or chloroethylamines—inhibited hexokinase when present in low concentrations, and suggested that vesication was due to this inhibition. The inhibition of mitosis is possibly connected with poisoning of enzymes of the same type either concerned in general metabolism, or possibly in the changes in the nucleic acids or enzymes concerned in phosphate transfer in nucleic acids of the chromosomes.

CYTOLOGICAL EFFECTS.

The cytological effects induced by chloroethylamines were studied in transplanted Walker rat carcinoma. The animals were injected intraperitoneally with 1 mg. per kg. body weight 4–10 days after tumour implantation, and killed at intervals of from 6 hours to 5 days after treatment.

The earliest abnormalities were seen 8 hours after injection. These included the formation of dumb-bell-shaped nuclei (Fig. 5), due to the fact that at telophase the daughter chromosomes could not separate and move to the opposite poles freely, owing to apparent stickiness of the chromosome ends (Fig. 4). Eighteen hours after injection chromosomes in dividing cells fragmented and mitosis remained incomplete (Fig. 6). This effect was most pronounced at 24 hours to 48 hours after treatment, when all dividing cells were abnormal and cell proliferation might be stopped completely. This inhibition lasted for 3–7 days, after which time normal cell division was resumed. When the dose was divided into two equal fractions and injected at 24-hour intervals, the cytological abnormalities were of the same kind as described above, but the damage in individual cells was less severe. Loss of chromosome segments from the nucleus leads to the death of the cell, and is also responsible for the arrest of tumour growth. Treatment of rats with Walker carcinoma with doses of 100 mg./kg. of dimethyl-β-chloroethylamine produced no abnormalities in the dividing cells of the tumour tissue examined 24 and 48 hours after treatment.

The biological effects of nitrogen mustards induced in experimental animals provide a rationale for employing them as chemotherapeutic agents in the treatment of tumours.

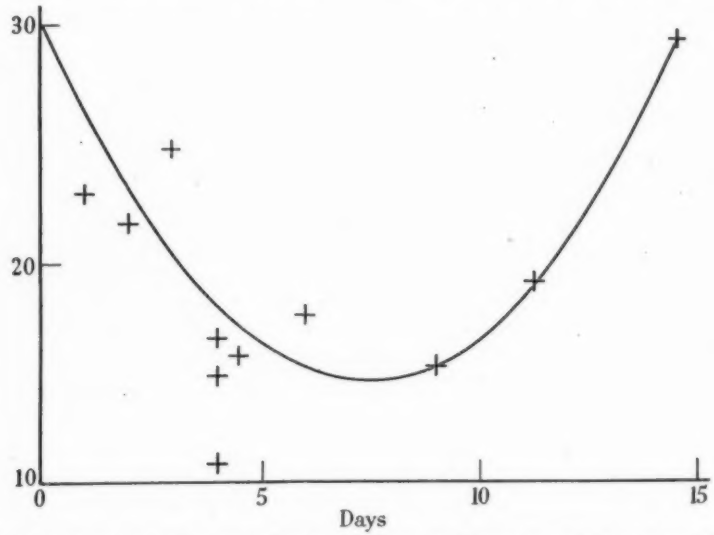


FIG. 7.—The glycolysis of Walker rat carcinoma tissue at different times following treatment of the rats with 1 mg. per kg. methyl-*bis*(β-chloroethyl)amine (HN2).

EFFECTS ON BRONCHOGENIC CARCINOMA.

During the past three and a half years some 60 cases of bronchogenic carcinoma unsuitable for other forms of therapy have been treated with various compounds. From 1944 to 1946, 22 patients received either urethane, stilboestrol, dienoestrol, dimethylaminostilbene or 4:4′-diaminodiphenylether. Symptomatic improvement was observed in 1 of the 15 patients receiving urethane, but none of the other drugs had any beneficial effect.

Rhoads (1946) reported a favourable but temporary effect in a case of anaplastic bronchogenic carcinoma receiving methyl-*bis*-(β-chloroethyl)amine, and suggested that further clinical trials were justified. During the first 10 months of 1947, 41 histologically proven cases of bronchogenic carcinoma have been given this compound. Of this group 15 have been referred from the Brompton Neoplastic Clinic, nine from the Royal Cancer Hospital (Free), and the remainder from physicians in other London hospitals.

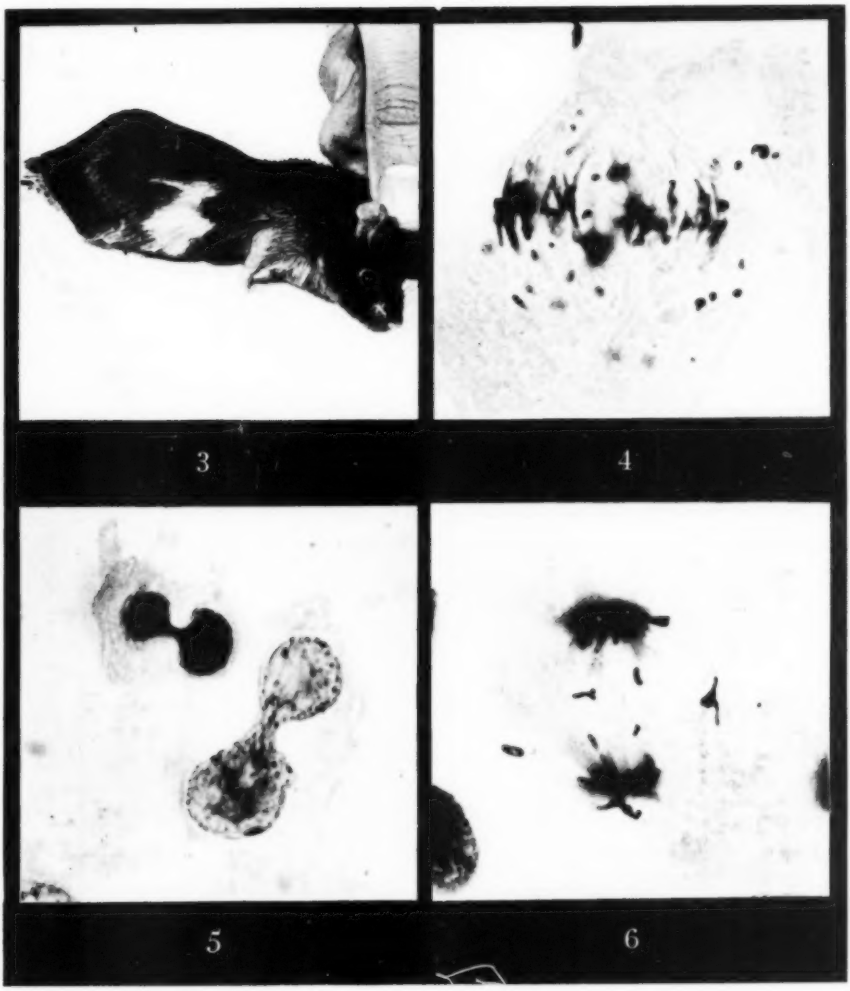
EXPLANATION OF PLATE.

FIG. 3.—Black mouse following three subcutaneous injections each of 0·004 mg. of methyl-*bis*(β-chloroethyl)amine (HN2), showing bleaching or "greying" of hair. Photograph taken six months after the last injection.

FIG. 4.—Cell of Walker rat carcinoma in mitosis 12 hours after injection of HN2. The nucleic acid charge of the chromosomes is altered, causing stickiness of chromosomes. × 1650.

FIG. 5.—Two cells of Walker rat carcinoma with dumb-bell-shaped nuclei, 24 hours after HN2. The daughter chromosomes did not separate, and the two daughter nuclei remain in association. × 1650.

FIG. 6.—Cell of Walker rat carcinoma in anaphase of mitosis 48 hours after HN2. Several of the chromosomes are fragmented, the fragments are left scattered in the cytoplasm and will be lost from the nuclei. × 1550.



3 4

5 6

Boyland, Clegg, Koller, Rhoden and Warwick.

Unsuitability for surgery implies that the growth is not resectable, and for radiation therapy that the patient is too ill for such treatment, that there is evidence of extension of the disease beyond the mediastinum, or that there is fever or pleural exudation. The prognosis in cases unsuitable for surgery and radiation therapy is indicated by the fact that of an untreated group of 50 clinically similar cases, one-third died within 1 month and none lived beyond 8.

In this group, one-third were dangerously ill, being bed-ridden with the disease. The sex incidence and frequency distribution of histological type were similar to those of a total group of 194 seen at the Brompton Neoplastic Clinic.

Thirty-three patients had no previous treatment. Of the remainder, 3 had exploratory thoracotomy, 1 had pneumonectomy with later appearance of secondaries, and 4 had received radiation therapy which had been either clinically unsuccessful, or had been followed in a few months' time by recurrence of symptoms and progression of the disease.

Form of treatment.

All patients in this group except 1 received methyl-*bis*-(β-chloroethyl)amine hydrochloride (HN2). A variant of this compound, dimethyl-β-chloroethylamine hydrochloride, was used in 3 other cases, reported here because of the favourable response in 1 case. The compound HN2 was given intravenously in a dose of 0·2 mg. per kg. on two consecutive days in 30 cases, in 10 of which treatment was repeated once or several times at 3-week or longer intervals depending upon the length of remission from symptoms. Two patients received 1 single injection of 0·2 mg. per kg., and two received 1 injection of 0·4 mg. per kg. Four patients received injections of 0·2 mg. per kg. at weekly or 10-day intervals for periods of up to 10 weeks. In the cases receiving the dimethyl compound, 3 mg. per kg. was given the first day and a dose of 5 mg. per kg. the following day.

Several of the ambulant cases received treatment as out-patients, and it would seem that there is no contraindication to such treatment, provided the patient can be returned to his home within an hour of the injection.

Adverse effects of treatment.

No serious reactions have been observed in this group. In almost all patients Table II) nausea and vomiting follow injection one to two hours later and

TABLE II.—*Adverse Effects of HN2.*

Total number of injections	152
Nausea	130
Vomiting	121
Anorexia	94
Weakness	15
Drowsiness	13
Light-headedness	7
Thrombophlebitis	4
Diarrhoea	2
Rigor	2

continue in most cases for 3–4 hours. In the majority anorexia persists for 24 hours. Feelings of weakness, drowsiness and lightheadedness may occur. In 1 patient such symptoms persisted for 1 week. Thrombophlebitis at the site of the injection, of no serious consequence, followed 4 of over 150 injections. Diarrhoea was remarked upon by 2 patients, and lasted for 3 and 24 hours respectively. Rigors lasting a few minutes have followed 2 injections.

With a dosage of 0·2 mg. per kg. on consecutive days the effects on the white cell count have not been serious. The majority of patients with advanced bronchogenic carcinoma have white cell counts above 10,000 per c.mm., and such patients do not develop the moderate to severe leucopenia observed in cases of Hodgkin's disease (ApThomas and Cullumbine, 1947). In only 2 of our patients has the white cell count fallen below 4000 per c.mm., in these cases to 1000 and 1800 with restoration to normal counts in 3 weeks' time. The first of these cases had had previous radiation therapy, and at the commencement of treatment had a white blood cell count of 4000 per c.mm. Haemorrhagic manifestations have not been observed in this group, nor has any significant change in haemoglobin been noted. Dosages of 0·2 mg. per kg. at 10-day intervals can be tolerated for periods of at least 10 weeks, as judged by the 4 cases we have treated over such a period. The presence of fever, pleural exudation or jaundice are not, in our experience, contraindications to the use of the drug.

The adverse effects of the dimethyl compound were observed in 3 patients, 2 of whom are not included in this group as their diagnosis was not histologically proven. Each of these patients had flushing at the time of injection. Nausea and vomiting were similar to that following the use of HN2, but neurological effects were seen. Patients stated that for several days they could not taste their food; one was confused and disoriented for 5 days, and another, besides being disoriented, was irrational and had incontinence of urine.

Effects of treatment.

Twenty-five of the cases reported in this group are still living and have been under observation from 4 to 10 months. Sixteen patients have died.

In estimating the effects of treatment this group may be divided into four classes:

(1) Showing symptomatic relief with objective signs of improvement (18).
(2) Showing symptomatic relief only (6).
(3) Showing objective signs of improvement with no symptomatic relief (1).
(4) Showing no apparent effect (16).

Remissions when they occur usually last 2 to 12 weeks. Further injections produced similar results in most cases. One patient was carried on for a period of 5 months with marked relief of pain, cough and dyspnoea and decrease of sputum for 18 to 21 days following each course of injections. He then showed no further response to the drug and died soon thereafter. A patient with remission of symptoms for 6 to 8 weeks after each treatment is still being observed after 9 months. One patient receiving dimethyl-β-chloroethylamine is still in remission 7 months after treatment and leading a normal life. Two other patients, however, not histologically proven, and therefore not included in this report, were also given the same drug with no apparent beneficial effect.

It is noteworthy that over half of the patients have had relief from their most distressing symptom. The effect on pain (Table III) is remarkable in some

TABLE III.—*Symptomatic Relief following HN2.*

Total number of cases	41
Symptomatic relief	24

	Number of cases.	Relief obtained.
Pain	23	17
Cough	25	16
General malaise	27	16
Anorexia	23	14
Dyspnoea	22	11
Hoarseness	8	0

cases, relief being experienced within 8 to 48 hours. The pain referred to as being "deep in the chest" is the type most commonly relieved, in contrast to the more localized pain of pleural origin.

Cough is often decreased in severity and may cease altogether. The relief of dyspnoea in some cases is almost complete—an observation very difficult to explain, for in such cases there may not be corresponding observable changes in the lungs.

Relief of general malaise and anorexia is also noted in over half the cases following the initial 1-or 2-day period of adverse effects. Improvement in hoarseness, secondary to involvement of the recurrent laryngeal nerves, did not occur in any of the cases.

Objective signs of improvement.

Approximately one-half of the patients (Table IV) had some measurable beneficial effect of treatment, and in all except one of these some symptomatic relief was also experienced.

TABLE IV.—*Objective Improvement following HN2.*

Total number of cases	41
Objective signs of improvement	19

	Number of cases.	Effects noted.
Decrease, caval obstruction	5	3
Decrease, sputum	30	18
Decrease, pleural exudate	13	5
Decrease, metastatic tumour size	10	4
Increase, weight	22	9
Radiological improvement	35	8
Remission, physical signs (chest)	39	9
Remission, neurological signs	10	1

Of 5 patients with obstruction of the superior vena cava, swelling of the arms disappeared in 1. In 2 others who were moribund at time of treatment regression of swelling to one-half the original size was noted within a week of treatment.

Sputum usually increases in amount for several days, and is said "to come up more easily." The amount may then fall off rapidly, in some cases from 90–120 ml. to 15 ml. or *nil* within a week. The sputum may also change in character from purulent to colourless.

Reabsorption of pleural exudate or decreased rate of formation has been seen in 5 of 13 cases. These observations are of interest in that most radiotherapists hesitate to give treatment to cases with pleural exudation because of the tendency to increase the amount of exudate.

Superficial metastases of measurable size decreased in 3 of 10 cases. A larger total number for such observations would have been obtained had not metastatic nodules been removed for microscopic study at various intervals following injection in 8 cases.

Twenty-two patients who had weight loss were weighed weekly following treatment. In 9 of these cases weight loss continued for approximately 1 week, following which a gain in weight occurred amounting usually to 2 to 8 lb. Four patients gained 10 to 15 lb. in 2 months, and 1 patient who lost 40 lb. in the 18 months prior to treatment with dimethyl-β-chloroethylamine has in 7 months regained all her lost weight.

Radiological evidence of decrease in size of pulmonary metastases and re-aeration of segments or lobes was observed in 8 of 35 cases. Such changes may happen within 5 to 7 days.

A favourable change in physical signs of the chest was noted in 9 of 39 cases, and included those cases with re-aeration of parts of the lung or decrease in pleural exudate.

As already mentioned, recovery of function from paralysis of the recurrent laryngeal nerves has not been noted. In one patient, however, with involvement of the twelfth cranial nerve, complete recovery of function occurred in 9 days and lasted for 7 weeks. Unfortunately, repetition of treatment was not possible in this case.

The appearance of primary tumours at bronchoscopy several days to several weeks following treatment has in the 6 cases observed been unaltered in 3 compared with previous examination. In 3 other cases the tumour area was said to be clearer and to bleed less readily, although a change in size was not observed.

Histological examination of primary or metastatic growth prior to and at various intervals following treatment was carried out in 8 cases. In all post-treatment specimens a great deal of necrosis was present, but one cannot say that such appearances are the result of therapy, for necrosis was seen in many of the pre-treatment specimens as well. Fragmentation of the chromosomes of dividing cells as described by Koller (1947) was not observed in 2 of the 8 specimens.

Case reports.

Case 1.—J. H—, male, aged 65. Seen first at neoplastic clinic December 13, 1946.

Symptoms: Cough, sputum, general malaise. Anorexia and dyspnoea on exertion for 4 months. Loss of weight—33 lb. in one year. Poor general condition; signs of consolidation and atelectasis right lower lobe. Small firm gland in right supraclavicular fossa. Weight 111 lb. Sputum, 90 ml. per day muco-purulent. X ray chest December 13, 1946, showed collapse and consolidation right lower lobe.

Bronchoscopy December 19, 1946. Ulcerated stricture of the right lower bronchus at the level of the middle bronchus almost obliterating the lumen. In view of the patient's age, poor general condition and the presence of small firm gland in the right supraclavicular fossa, operation contraindicated.

Pathological report: Poorly differentiated squamous carcinoma.

The presence of a supraclavicular gland made this patient unsuitable for X ray therapy and he was referred for chemotherapy. Two doses of methyl-*bis*-(chloroethyl)amine (0·2 mg. per kg.) given on two consecutive days were followed by nausea and vomiting after each injection. Immediately after the first injection the patient had a rigor for 5 minutes. Temperature 101°. Forty-eight hours after second injection the cough decreased, dyspnoea was much improved and the sputum decreased to 15 ml. per day. Appetite improved, and following initial loss of weight of 3 lb. he gained 8 lb. in one month. The cough and sputum started to increase again in 8 weeks' time but dyspnoea did not return. Radiological examination revealed no changes in the lungs following therapy. Treatment has been repeated on two occasions since with corresponding remission of symptoms and decrease of sputum. Present weight is 108 lb. and general condition remains about the same as when first seen, there being no clinical or radiological evidence of progression of the disease.

Case 2.—L. P—, female, aged 47. Admitted to the Royal Cancer Hospital for treatment by Mr. R. W. Raven, April 24, 1947.

Symptoms: Cough with sputum, pain in the chest and dyspnoea 16 months. Loss of weight—40 lb. in 16 months. Patient moribund and emaciated, lying curled in bed without any interest in surroundings. Signs of collapse of right lung with mediastinal shift. Pulse 100. Respirations 24 per minute. Weight 81 lb. Sputum 30 ml. purulent and foul-smelling. Hb, 55 per cent. R.B.C., 3,800,000. W.B.C., 12,600.

X ray of chest April 8, 1947. Heart, mediastinum and trachea displaced to the right; appearance of atelectasis with probable cavitation near 2nd right rib. Bronchoscopy report from Harefield in April, 1946. Carina broadened and invaded on the right by a large nodular friable mass completely occluding the right main bronchus.

Pathological report: Adenocarcinoma with numerous mitotic figures.

Treatment: Dimethyl-chloroethylamine 2 mg. per kg. first day and 5 mg. per kg. second day. Flushing was noticed immediately after each injection. Nausea and vomiting were slight, and there was a feeling of numbness and "largeness" of the tongue with loss of taste for four days. Within one week pain and cough had disappeared. Dyspnoea at rest disappeared in two weeks. The sputum at first increased in amount and lost its bad taste, then decreased, with the cough finally disappearing. General condition slowly improved and in three months the patient was up. Weight steadily increased until 40 lb. was gained. Haemoglobin rose to 87 per cent. The only symptoms at present are a slight cough with a little colourless sputum and dyspnoea on exertion. The X ray appearances are unchanged, but the patient is leading a normal life and looking after her own home.

DISCUSSION.

The findings are of some theoretical interest in that a demonstrable clinical effect of the chloroethylamines on bronchogenic carcinoma in man has been

established. Other workers have been unable so far to demonstrate a corresponding effect on other types of carcinoma in human patients. This probably indicates some tissue specificity of action. It must be remembered, however, that patients are acutely conscious of symptoms referable to the chest and of their degree of severity. It may be that in bronchogenic carcinoma a slight diminution in size of a tumour or amount of tissue reaction around it is sufficient to afford considerable relief from the distressing symptoms. A corresponding effect in a tumour of the breast might reasonably be expected not to be noticed by the patient. In support of such a suggestion is the fact that a similar relief of respiratory symptoms following chloroethylamine without radiological evidence of changes in the lungs has been observed in a case of carcinoma of the breast with pulmonary metastases. On the other hand, metastatic nodules of bronchogenic carcinoma have been seen to decrease in size or even disappear, whereas such changes have not been observed with other types of carcinoma similarly treated at the Royal Cancer Hospital.

From a therapeutic standpoint it can be stated that little has been added to the satisfactory handling of cases of carcinoma of the lung. Impressions based on this study are that the use of the drug may be of some value in cases unsuitable for other forms of therapy, where relief of distressing symptoms may make the patient's remaining days more tolerable. Such advantages, however, should be weighed against the probable amount of discomfort which will be caused by treatment.

There is no definite evidence that the drug alters the progressive course of the disease in the selected group of patients studied, although in the case histories presented it would seem that treatment had a very favourable result, which would not have been expected to occur by chance alone.

The results of further investigations with different dosages of these drugs and variations in the schedule of administration, of the effects in a group of less advanced cases and in a series at present being treated by combination of the drug with radiation therapy, should establish whether these compounds are of more than temporary palliative value. If a palliative remedy is employed then it should be employed continuously at the optimum level of dosage. This dosage remains to be determined.

SUMMARY.

Methyl-*bis*(β-chloroethyl)amine hydrochloride (HN2) and *tris*(β-chloroethyl)-amine (HN3) produce many effects similar to those caused by X rays, including the greying of hair of mice at the site of injections.

Injection of HN2 or HN3 in adequate doses into animals with particular tumours causes temporary inhibition of tumour growth. This inhibition is accompanied by a reduction in tissue glycolysis. These substances have nucleotoxic actions, as shown by apparent increased stickiness and fragmentation of chromosomes which lead to the death of the injured cell. By repeating the treatment at suitable intervals the life of animals with tumours can be prolonged, but cessation of treatment leads to recurrence of tumour growth.

Forty-one cases of histologically proven carcinoma of the bronchus unsuitable for other forms of therapy have been treated with the chloroethylamines. No serious adverse reactions have been noted. Symptomatic relief and objective signs of improvement were noted in approximately one-half of the cases.

Remissions, when occurring, have lasted usually from two weeks to three months. The theoretical interest of the apparent specificity of chloroethylamine in bronchogenic carcinoma and the indications for its possible use as a palliative measure in advanced cases of the disease are discussed.

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REFERENCES.

ApThomas, I., and Cullumbine, N.—(1947) *Lancet*, i, 899.
Boyland, E.—(1941) *Biochem. J.*, **35**, 1283.—(1946) *Brit. J. Pharmacol. Chem.*, **1**, 247.
Dixon, M., and Needham, D. M.—(1946) *Nature*, **158**, 432.
Gilman, A., and Philips, F. S.—(1946) *Science*, **103**, 409.
Goodman, L. S., Wintrobe, M. M., Dameshek, W., Goodman, M. S., Gilman, A., and McLennan, M. T.—(1946) *J. Amer. med. Ass.*, **132**, 126.
Jacobsen, L. O., Spurr, C. C., Burton, B. S. G., Smith, T. R., Lushbaugh, C., and Dick, G. F.—(1946) *Ibid.*, **132**, 263.
Koller, P. C.—(1947) *Brit. J. Radiol. Supplement*, **1**, 84.
Rhoads, C. P.—(1946) *J. Amer. med. Ass.*, **131**, 656.
Wilkinson, J. F., and Fletcher, F.—(1947) *Lancet*, ii, 540.

CULTURE OF HUMAN LEUKAEMIC BLOOD CELLS *IN VITRO*; TECHNIQUE AND THE GROWTH CURVE.

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The aim of the work reported in this paper was to perfect a simple and reliable technique for the culture of human leukaemic blood cells, and with it to construct a quantitative base line which would later be available for testing the effects *in vitro* of therapeutic measures such as ionizing radiations or chemical agents. These effects express themselves as changes in the number as well as the appearance of various cell types. Furthermore, the effect on cells in mitosis is of particular interest and importance.

The results which are here reported provide the necessary controls for experiments with therapeutic agents, by establishing the rate of growth and decay

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of untreated leukaemic cells observed *in vitro*. Only the quantitative aspects of the subject will be dealt with here; the cytological findings will be described in a separate paper.

Normal and leukaemic blood and bone marrow have been studied in tissue cultures by many workers since the publication of the first report on tissue culture of leukaemic blood by Awrorow and Timofejewskij (1914). Among other investigations carried out in recent years the following should be mentioned, as they deal wholly or in part with culture of leukaemic blood or marrow: papers by Barnes and Furth (1937) and Bichel (1940) on mouse leukaemia; Doljanski and Pikovski (1941) on fowl leukosis; and Fieschi and Astaldi (1946), Isräels (1940*a*, 1940*b*), Osgood and Brownlee (1936, 1937, as well as many later papers from the same laboratory), Pierce (1932, 1942), Schrek (1946*a*, 1946*b*), and Wallbach (1936), on human leukaemia. Two main culture methods have been employed: On the one hand, cultivation of solid fragments of marrow or of coagulated buffy coat from the peripheral blood, either in hanging drops or in tubes, which has been favoured by most workers; on the other, the fluid method originated by Osgood and used later by Isräels and recently by Schrek. A third alternative is cultivation on the chick chorio-allantoic membrane, as suggested by Pierce (1942).

In the investigation of quantitative changes in a given cell population the fluid method offers certain advantages, as it allows of total, as well as of differential cell counts. It is therefore possible to determine the absolute numbers of the various cell types, as well as of mitoses, present at any moment, to note their increase or decrease under experimental conditions, and to observe changes under the influence of therapeutic agents. While much work has been done on these lines on bone-marrow cells, mainly by Osgood and his collaborators, few similar data exist for leukaemic blood. Schrek, one of the few investigators in this field, employed a saline culture medium which did not allow of cell division, so that his valuable findings are not entirely comparable with those obtained by the use of an enriched medium.

TECHNIQUE.

The technique has been modified from that described by Osgood and Brownlee, the main changes being a simplification of the composition of the culture medium and of the procedure in setting up the cultures. The principle of the method is the distribution of small equal quantities of blood cells, suitably diluted with culture medium, over a series of culture tubes, the whole process being carried out by means of hypodermic syringes.

Apparatus.

The following are required: Screw-cap bottles of the McCartney type, to hold 25–30 ml.; test-tubes $1\frac{1}{2} \times 7$ cm.; culture tubes (round-bottomed tubes of hard glass, $2\frac{1}{2} \times 7\frac{1}{2}$ cm., with a neck which can be fitted with vaccine caps); glass caps to cover the vaccine cap and fit the shoulder of the culture tube (both the last items can be easily made by any competent glass-blower); rubber vaccine caps to fit the culture and small test-tubes; 50 and 250 ml. conical flasks, with rubber bungs and glass caps to fit their tops; 1, 2, 10 and 30 ml. all-glass syringes with suitable needles.

All the glassware should be cleaned by the method in general use in tissue-culture laboratories (Cameron, 1935). The vaccine caps are washed, and as rubber is toxic to living cells, they are then coated with a very thin layer of paraffin wax, by dipping them into a saturated solution of paraffin in petrol ether and wiping off excess after withdrawal. The culture tubes are assembled as shown in Fig. 1, a No. 1 hypodermic needle being passed through each vaccine

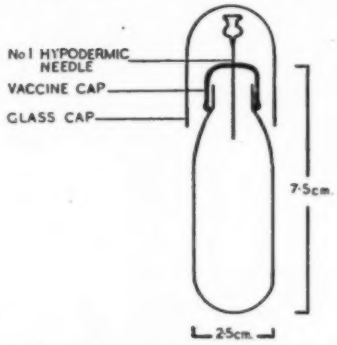

FIG. 1.—Culture tube ready for introduction of medium.

cap ; the short test-tubes are similarly capped ; they and the screw-cap bottles are fitted with needles and cellophane caps, and all apparatus is sterilized by autoclaving.

Culture medium.

This consists of equal parts of Ringer solution at pH 7·3 and of unfiltered reconstituted adult human plasma, as supplied in dried form by the National Blood Transfusion Service. Preliminary experiments showed that there was no significant difference between the results obtained by the use of human placental serum as recommended by Osgood, fresh adult serum and dried and reconstituted plasma. The latter offers the advantage of ready availability in sterile form, and of constant composition. After reconstitution it has been kept for weeks frozen at −20° C., without a detectable change in its properties.

Setting up of cultures.

Venous blood is taken under sterile precautions into screw-cap bottles containing enough heparin to give a final concentration of 1:15,000. The quantity of blood required varies between 5 and 30 ml., according to the height of the patient's leucocyte count and the number of tubes to be set up. The bottle is allowed to stand in the incubator at 37° C. for ½ to 1 hour, when most of the erythrocytes will have settled, the leucocytes and platelets remaining suspended in the supernatant layer. This is now removed and transferrred to a short test-tube, in which it is centrifuged for 1 to 2 minutes at 500 r.p.m. The supernatant plasma is removed, and the cells are washed once in Ringer solution. All the manipulations are carried out by means of syringes, none of the containers being opened in the process. Meanwhile, a suitable quantity of culture medium has

been put into a conical flask, and the cells are now added to it, the aim being to give a final concentration of about 2000 leucocytes per c.mm. Some practice is required in judging the correct volume of cells to be added. Three consecutive counts are done on the cell suspension, and when the mean reveals a suitable cell concentration, a quantity of 2 ml. is introduced into each culture tube, care being taken to avoid settling of the cells in the conical flask by shaking the latter at frequent intervals. The No. 1 needles are now removed from the culture tubes, which are incubated at 37° C.

The process of setting up 36 to 48 cultures by this method requires about 1½ hours from the moment of taking the blood, working with an assistant. There is always the danger of contamination of the cultures, although the "closed" method minimizes it, and if working conditions should be unfavourable, it may be necessary to add penicillin to the cultures in a final concentration of 100 units per ml. There is good evidence (Gey, Gey, Inui and Vedder, 1945; Bucher, 1946) that crystalline penicillin has no deleterious influence on tissue cultures. Streptomycin has also been employed, but it may possibly damage the cells.

Changing of culture medium.

In the early experiments the culture medium was changed every 2 or 3 days, as recommended by Osgood (1939) and Isräels, but it was found that replacement of the old by an exactly equal quantity of fresh medium was difficult and constituted a source of errors in counting, and that infections were easily introduced in the process. Experiments were then made in which the medium was left unchanged, and no significant difference was noted in the results. This is not unexpected, for in the cultures the volume of the medium exceeds that of the cells by a factor of 10^3 or more, so that it is difficult to imagine the cells being adversely influenced either by exhaustion of the medium or accumulation of metabolites. On the other hand, results have never been satisfactory when the thickness of the fluid layer exceeded 1 cm., owing, presumably, to the slow diffusion of oxygen to, or of CO_2 from the cells.

Counting.

After thorough shaking of the tubes, counts are done on unstained cells in undiluted suspension, using Thoma or Neubauer chambers. Difficulties arise in the later days of culture owing to degeneration of some of the cells. Many of these become completely non-refractile and ghost-like, and it is this class of cell which loses practically all power of staining in smears. It will therefore not be counted in differential counts, and a grave source of error will thereby be introduced. For this reason, "ghost cells" are not included in total counts, but only cells showing at least a remnant of refractility.

The parameter count of each tube is calculated by counting all leucocytes in 1 $mm.^2$ of the ruling, multiplying by 10 and rounding off to the nearest 100.

When the volume of fluid in each culture tube is small, as in these experiments, it is unsatisfactory to remove repeated samples for counting, as differences may thereby be introduced in the quantities of fluid remaining in the tubes. It should be more desirable to use a fresh part of the series of tubes for each count, discarding it after counting, provided that it can be shown that the distribution of counts over the total series of tubes is approximately normal, and that the

differences arising between individual tubes in the course of the experiment remain reasonably small. The following experiments were designed to test these questions, duplicates being done in each case :

Experiment 1.

Blood was obtained and treated as described, until the final leucocyte suspension had been made. A series of 36 counts was then done on it, the same syringe being used for all. The results are shown in Fig. 2, from which it can be seen

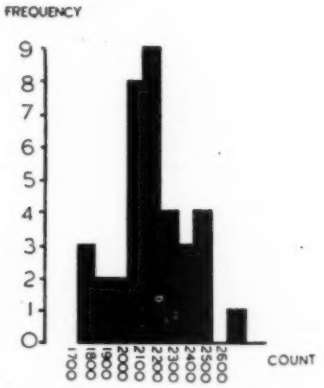

FIG. 2.—Frequency distribution of 36 counts of a given W.B.C. suspension. Mean = 2090 ± 208/c.mm.

that the distribution is approximately normal. The large standard deviation of ± 10 per cent should be noted. This is chiefly due to the fact that in the initial stages of the culture there occurs a remarkable clumping of the leucocytes, agglutinates of three to five cells being often observed. This phenomenon, which is variable in degree, has largely disappeared after 24 hours. It is probably analogous to " leukergy " observed in inflammatory states by Fleck and Murczynska (1947).

Experiment 2.

A series of 36 cultures was set up as described. The mean of three counts of the final cell suspension, before distribution, was taken as the 100 per cent initial value for all tubes. After 24 hours, six tubes were removed and five counts done on each. Six fresh tubes were similarly counted on each of the next 3 days. No count was done on the fifth day, and a single count on each of the remaining twelve tubes on the sixth day. The standard errors between individual counts and between tubes were calculated for each of the first 4 days. The results are shown in Table I, and graphically in Fig. 3. Table I shows that after the first day the standard error between tubes became somewhat large. This was often due to one or two individual tubes getting " out of step " with the others, probably owing to some special factor operating only in them ; examples are tubes 12 on the second and 24 on the fourth day. The standard

TABLE I.—*Daily Counts and Standard Error of a Sample Culture of Blood (Chronic Myeloid Leukaemia). Multiply Figures by 100. Initial Count : 2130 ± 57 W.B.C. per c.mm.*

Day.	Count No.	Tube number. 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	Mean.	Standard error of mean. Betw. counts.	Betw. tubes.
	1	20	22	19	18	22	19																															20·0		
	2	21	23	20	23	18	21																															21·0		
1	3	17	20	19	23	22	15																															19·3	±0·77	±1·33
	4	18	20	20	22	20	21																															20·2		
	5	18	22	18	25	23	21																															21·2		
	1							13	21	16	12	13	7																									13·7		
	2							19	21	16	14	12	5																									14·5		
2	3							20	17	20	12	13	6																									14·7	±0·68	±5·00
	4							20	17	17	14	12	4																									14·0		
	5							15	18	18	11	11	5																									13·0		
	1													4	5	7	9	14	10																			8·2		
	2													3	5	5	10	10	12																			7·5		
3	3													5	6	6	10	11	12																			8·3	±0·62	±3·13
	4													6	4	4	9	11	9																			7·1		
	5													5	4	6	9	14	10																			8·0		
	1																			6	8	8	5	4	2													5·5		
	2																			4	6	8	4	3	1													4·3		
4	3																			5	9	7	8	3	2													5·7	±0·59	±2·64
	4																			4	10	7	8	2	2													5·5		
	5																			6	9	8	3	2	1													4·8		
6																										1	1	1	1	1	1	0	2	2	2	2	3	1·4		±0·9

error between counts (the experimental error) remained consistently smaller than that between tubes. In spite of the large size of the latter, the trend of the curve is clear from Fig. 3. It is to be noted that results are consistent with an exponential decrease with time of the initial cell count, after the first day of culture.

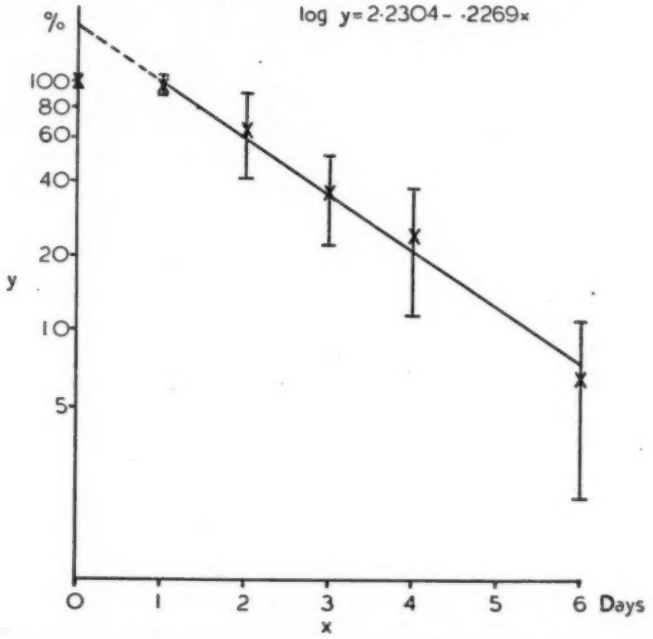

FIG. 3.—Mean daily counts of a sample culture of blood (chronic myeloid leukaemia), expressed as per cent of initial count drawn on logarithmic scale.

In the light of these two experiments, the following routine procedure has been thought justifiable: The mean of three counts of the original cell suspension is taken as the 100 per cent initial value for all tubes. On each subsequent occasion three separate counts are done on each of three fresh tubes, and the mean and standard error between tubes are calculated for all nine counts. The growth curve is constructed from the resultant points.

Differential and mitotic counts.

After the total counts have been done, all the fluid remaining in the culture tubes is transferred to Wintrobe tubes and centrifuged for one minute at 500 r.p.m. The supernatant is removed, and the cells are resuspended in approximately twice their own volume of supernatant and mixed as thoroughly as possible. Smears are then made and stained as desired. Differential counts of 500 cells are done on one smear from each tube (1500 for the three tubes routinely used); total and differential counts are also done of all mitoses found in 1000

immature cells in each of the three smears. Means are calculated from the three individual counts and used for constructing the growth curve.

In these differential counts a simplified nomenclature of the myeloid series has been used, cells being divided into the three broad classes of myeloblasts, myelocytes and polymorphonuclears (eosinophils and basophils being counted separately). The term " immature cell " comprises myeloblasts and myelocytes, the latter being arbitrarily defined as complying with two or all of the three following characteristics : total cell size exceeding that of a normal polymorphonuclear by 1/3 or more ; nucleus oval and only slightly indented ; cytoplasm basophilic. The term " polymorphonuclear " thus comprises most metamyelocytes, and all juvenile and stab forms in addition to completely mature cells. The dividing line has been drawn so as to separate those cells considered capable of mitotic division from the rest ; it is, however, realized that no absolutely certain distinction is possible.

RESULTS.

This report is based on the findings in 31 cultures of blood from 11 patients with chronic myeloid leukaemia. Clinical details of these patients are available if required. Six cultures of blood from patients with chronic lymphatic and with acute leukaemia were also made, but the findings were not yet sufficiently clear to warrant publication. Some of the patients with chronic myeloid leukaemia were untreated, while others had had various forms of therapy.

Cultures were either continued until all cells had died, or they were deliberately abandoned at an earlier period. When they were continued, there was always a gradual decrease in the total counts until death of the last cells occurred, usually between the sixth and the ninth day of culture. No significant increase in total counts has ever been observed.

Of especial interest is a group of nine cultures, selected because the cells lived for at least 7 days. The patients from whom the cells in this group were taken either had not been treated or had received no recent treatment. The daily mean counts of the individual cultures are given in Table II. These results

TABLE II.—*Daily Counts of Nine Cultures of Blood (Chronic Myeloid Leukaemia) Expressed as Per Cent of Initial Counts.*

Day.	Culture Number.									Mean.
	1	2	3	4	5	6	7	8	9	
0 .	100	100	100	100	100	100	100	100	100	. 100
1 .	76	117	103	87	102	104	78	107	98	. 97 ± 13·7
2 .	62	104	94	72	88	82	70	82	101	. 84 ± 13·7
3 .	66	88	67	74	—	74	62	68	72	. 71 ± 7·9
4 .	65	56	—	52	72	—	—	57	74	. 63 ± 9
5 .	50	47	54	26	60	65	40	50	45	. 49 ± 11·3
6 .	—	—	47	23	46	78	—	—	—	. 46 ± 22·7
7 .	35	47	46	—	18	44	28	32	23	. 34 ± 10·9

are represented graphically in Fig. 5, which shows that after the first day the fall in the cell count is consistent with an exponential decrease with time. This finding is not unexpected, but its significance must not be over emphasized. It is worthy of comment that the experimental values, including the initial point, are also consistent with a linear decrease with time ($y = 103{\cdot}1 - 9{\cdot}88\,x$).

However, the individual experiments show that the results are not consistent with a linear decay, but are satisfactorily accounted for by an exponential decrease with time, after the first day (Fig. 3 and 4). The line shown in Fig. 5

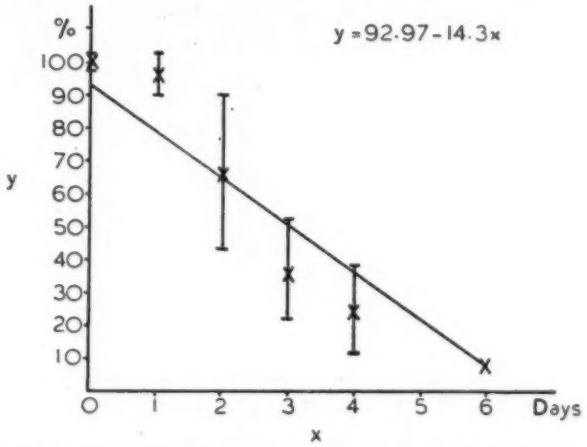

FIG. 4.—Mean daily counts of a sample culture of blood (chronic myeloid leukaemia), expressed as per cent of initial count drawn on linear scale.

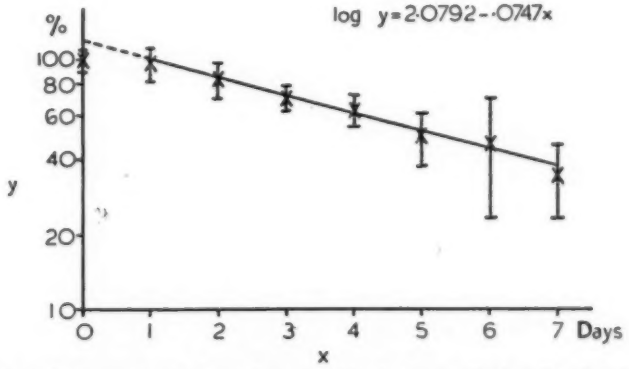

FIG. 5.—Mean daily counts of nine cultures of blood from patients with chronic myeloid leukaemia, expressed as per cent of initial count.

is very similar to one given by Schrek (1946*a*), who estimated the survival of blood cells from patients with chronic myeloid leukaemia in " phosphate ringer solution," the main difference being the steeper slope of Schrek's line.

As it appeared desirable to follow in greater detail the fate of the cells during the first 48 hours of culture, experiments were made in which the cells were counted at 8, 24 and 48 hours from the time at which the cultures were set up ($1\frac{1}{2}$ to 2 hours should be added if the time from the taking of the blood is required).

Parallel cultures were made in medium containing rat embryo extract (E.E.) in a final concentration of 5 per cent. The findings in one of these experiments are shown in Fig. 6 and 7. From these it is seen that while the fluctuations

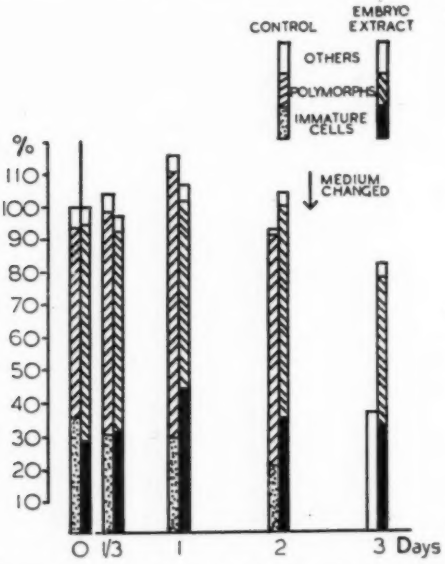

FIG. 6.—Effect of 5 per cent rat embryo extract on culture of blood (chronic myeloid leukaemia). Total and differential counts expressed as per cent of initial count.

of the total count in the two series are probably not significant, they certainly do not follow a straight line. Of greater interest is the behaviour of the immature cells, in which a striking difference between the two series is apparent. While there is a gradual decrease of the number of immature cells in the control cultures, there is a significant rise in the E.E. cultures, which attains a peak at 24 hours and is followed by a decline. At the same time, differences occur between the mitotic rates of the two series, the curve for the E.E. cultures showing a peak of more than four times the height of that in the control cultures.

Results similar to these have been found in all cultures in which differential counts were done. When the basic medium was used, a mitotic rate of between 0·2 and 0·6 per cent has always been observed during the first 24 hours; differential counts, while revealing minor differences between cultures, have in no case shown a significant increase in the number of immature cells, unless E.E. was present. After the second day there has been remarkably little change in the differential counts.

DISCUSSION.

The shape of a growth curve is influenced by three main factors, apart from those operative through the experimental error: the rate of division of the immature cells, that of their maturation, and that of the decay of the mature

cells (as well as of the immature ones in the later stages, when maturation has ceased). These factors vary from case to case in their relative importance, so that a culture from each individual blood will possess its own characteristic curve, and will be comparable only with other cultures made from the same blood. For instance, a blood may contain many immature cells, and owing to their proliferation the total count may well decrease less rapidly than in another blood containing mostly polymorphonuclears.

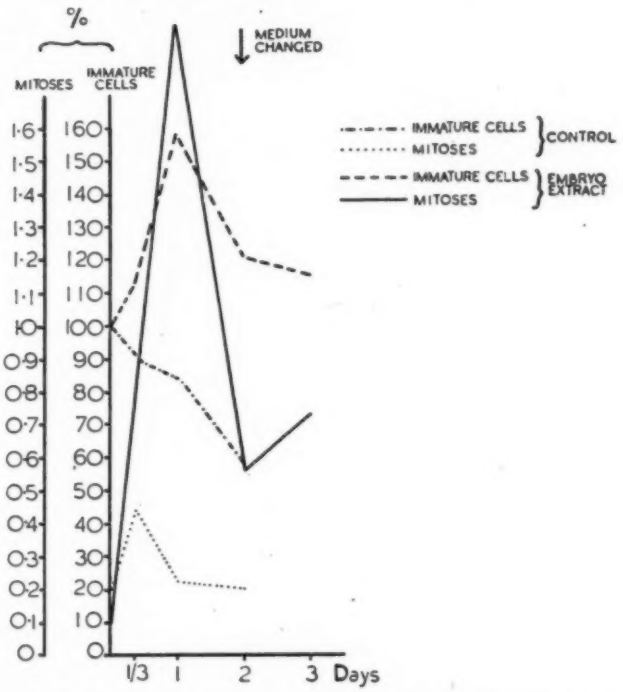
%
MEDIUM CHANGED
MITOSES
IMMATURE CELLS
1·6 1·5 1·4 1·3 1·2 1·1 1·0 0·9 0·8 0·7 0·6 0·5 0·4 0·3 0·2 0·1 0
160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
IMMATURE CELLS
MITOSES
CONTROL
IMMATURE CELLS
MITOSES
EMBRYO EXTRACT
1/3 1 2 3 Days

FIG. 7.—Effect of 5 per cent rat embryo extract on culture of blood (chronic myeloid leukaemia). Immature cells and mitoses expressed as per cent of initial count.

The rate of mitotic division increases rapidly during the first few hours of culture. Indeed, while mitoses are very rarely found in the peripheral blood of patients with chronic myeloid leukaemia, even after only two hours in the culture medium 0·1 to 0·2 per cent of immature cells are in mitosis. In the basic medium a peak of 4 to 6 mitoses per 1000 immature cells is reached after about 8 hours; this may be compared with Moeschlin's (1947) figure of 4·2 mitoses per 1000 immature cells in the bone marrow of untreated chronic myeloid leukaemia. If E.E. is added to the medium, the number of mitoses increases considerably beyond the eighth hour, and a peak is reached between the 24th and 36th hour.

It is difficult to obtain proof of maturation *in vitro*, although both the numerical and cytological findings suggest that it occurs. In basic medium

there is usually a definite increase in the total number of polymorphonuclears during the first 2 days, as for instance in the case shown in Fig. 6; the excess polymorphonuclears can have only been formed from immature cells, and the decrease in the number of the latter is explained by their proliferation failing to keep pace with maturation. After the second day, the process is less important and soon stops, mature and immature cells decaying approximately exponentially at the same rate, as shown in Fig. 3 and 5.

Death of the polymorphonuclears occurs from the earliest stages of culture. The average lifetime of these cells is given by Osgood (1937) as 61 hours, but this figure is probably not universally applicable. In the present experiments the half-life of all cells was approximately 4 days (Fig. 5); the length of survival of polymorphonuclears appeared reduced by the addition of E.E., a well-known metabolic as well as mitotic stimulant.

The evidence provided in this paper suggests that in fluid cultures the rate of proliferation of the cells decreases rapidly during the second 24 hours of culture and that it, as well as maturation, is of little importance after the second day in influencing the shape of the growth curve, which thenceforth becomes determined principally by degenerative processes. It is reasonable to assume that cells, when withdrawn from the body, are endowed with a residuum of substances promoting growth, and that these together possibly with a withdrawal of inhibitory factors operative in the body, are responsible for the initial mitotic activity in cultures. This residual "energy" is, however, rapidly exhausted, unless replenished by additional factors present, for instance, in E.E. The work of Waymouth (1947), and of others, suggests that the growth factor in E.E. may be a ribo-nucleoprotein, and experiments are now in progress designed to co-ordinate the growth curve with the nucleic acid metabolism of the cells.

The action of ionizing radiations, as well as of many chemotherapeutic agents, is primarily on mitotic cells. In investigating these actions, every effort should therefore be made to provide the maximum number of mitoses in the test object. Thus in fluid cultures the addition of E.E. is indicated in all experiments designed to test therapeutic agents. Furthermore, as mitoses are infrequent and degenerative processes normally predominate after the second day, an unnecessary effort is involved in continuing the cultures beyond it.

SUMMARY.

A simple method of making tissue cultures of human leukaemic blood cells in a fluid medium is described.

Difficulties encountered and precautions to be taken in doing total, differential and mitotic counts are mentioned.

The addition of embryo extract greatly increases the number of mitoses found during the first 2 days in culture, and is recommended for all experiments designed to test therapeutic agents.

After the second day of culture the growth curve is determined mainly by degenerative processes in the cells; no useful purpose can be served in therapeutic tests if the cultures are prolonged beyond the first 48 hours.

I wish to express my thanks to the Saltwell Trustees for providing financial support for this investigation; to Professor J. S. Mitchell, for his constant help with the design of experiments and the preparation of this paper; to Dr. Martin

Hynes, for assistance with statistical problems; and to Professor Sir Lionel Whitby, Professor L. J. Witts, and Drs. L. B. Cole, F. B. Parsons and Ff. Roberts, for allowing me access to patients under their care.

REFERENCES.

AWROROW, P. P., AND TIMOFEJEWSKIJ, A. D.—(1914) *Virchows Arch.*, **216**, 184.
BARNES, W. A., AND FURTH, J.—(1937) *Amer. J. Cancer.* **30**, 75.
BICHEL, J.—(1940) *Arch. exp. Zellforsch.*, **24**, 27.
BUCHER, O.—(1946) *Schweiz. med. Wschr.*, **76**, 290 and 375.
CAMERON, G.—(1935) 'Essentials of Tissue Culture Technique,' p. 21. New York (Farrar & Rinehart).
DOLJANSKI, L., AND PIKOVSKI, M.—(1941) *Cancer Res.*, **1**, 205.
FIESCHI, A., AND ASTALDI, G.—(1946) 'La Cultura in Vitro del Midollo Osseo.' Pavia (Tipografia del Libro).
FLECK, L., AND MURCZYNSKA, S.—(1947) *Texas Rep. Biol. Med.*, **5**, 156.
GEY, G. O., GEY, M. K., INUI, F., AND VEDDER, H.—(1945) *Gibson Island Conf. Monograph*, p. 321.
ISRÄELS, M. C. G.—(1940*a*) *J. Path Bact.*, **50**, 145.—(1940*b*) *Ibid.*, **51**, 235.
MOESCHLIN, S.—(1947) *Helv. med. Acta*, **14**, 279.
OSGOOD, E. E.—(1937) *J. Lab. clin. Med.*, **24**, 954.—(1939) *J. Amer. med. Ass.*, **109**, 933.
Idem AND BROWNLEE, I. E.—(1936) *Ibid.*, **107**, 123.—(1937) *Ibid.*, **108**, 1793.
PIERCE, M.—(1932) *Arch. Path.*, **14**, 295.—(1942) *Ibid.*, **34**, 538.
SCHREK, R.—(1946*a*) *Radiology*, **46**, 395.—(1946*b*) *J. cell. comp. Physiol.*, **28**, 277.
WALLBACH, G.—(1936) *Arch. exp. Zellforsch.*, **18**, 315.
WAYMOUTH, C.—(1947) *Proc. Soc. exp. Biol. N.Y.*, **64**, 25.

CULTURE OF HUMAN LEUKAEMIC BLOOD CELLS *IN VITRO*. NORMAL AND ABNORMAL CELL DIVISION AND MATURATION.

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THIS paper describes the fate of human leukaemic blood cells in an artificial fluid culture medium. A previous paper (Gunz, 1948) has dealt with the numerical aspect of the problem, outlining the increase and decrease of the counts of mature and immature cells from which can be traced the growth curve of the cultures, and discussing the factors which influence its shape. It is now proposed to follow in detail the changing appearances of the various cell types, as seen in stained films, and to attempt an interpretation of some of the cytological findings. The account will be confined to cultures of blood from patients with chronic myeloid leukaemia, as not enough cultures of other types of leukaemia have yet been available to permit a general description.

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TECHNIQUE.

A full account of the technique has been given in the above-mentioned paper, to which reference for details should be made. Briefly the method of culture is a modification of that first suggested by Osgood and Brownlee (1936, 1937), and consists of the separation of the leucocytes of heparinized venous blood from the red cells and plasma, of their suspension in a culture medium made up of equal parts of dried and reconstituted unfiltered adult human plasma and Ringer solution at pH 7·3, and of the distribution of the mixture into a series of culture tubes, the whole process being carried out by a " closed " technique with sterile hypodermic syringes and needles. In certain conditions the addition of embryo extract (E.E.) to the medium is desirable, as it has been shown that the number of mitoses in immature cells is thereby greatly increased. The cultures are inspected and counted at suitable intervals, and individual tubes are discarded after one count in triplicate. The medium is left unchanged throughout each experiment.

Films of concentrated cell suspensions made after gentle centrifugation were in the present series stained by a combined Leishman-Giemsa stain, and by Feulgen's method. The former, for which the formula was kindly supplied by Dr. M. W. A. Gatman,* was used because, when suitably buffered (pH 6·81–6·85), it gave more uniform results than the usual Romanowsky stains, without, at the same time, giving rise to any unaccustomed appearances in the cells. The Feulgen-stained smears were always confirmatory of the others, and will not be separately described. Differential counts were done on 500 leucocytes, and mitotic counts on 1000 immature cells from each smear.

RESULTS.

This report is based on the findings in 31 cultures of blood from eleven patients with chronic myeloid leukaemia. Some of the patients were untreated, while others had had various forms of therapy. About 500–600 Leishman-Giemsa-stained films were studied in the course of these experiments.

The appearances described are those found in the basic culture medium. The addition of 5 per cent rat E.E. causes few differences, apart from raising the number of mitoses. The only divergent feature is the appearance of increased numbers of degenerate polymorphonuclears, probably owing to the metabolic stimulant action of the E.E.

From the cytological point of view, the life of each culture of leukaemic blood may be divided into two phases. The first, during which there is evidence of multiplication, and to a less extent of maturation, of the immature cells, reaches a climax during the first day, and comes to an end some time during the second 24 hours. The second phase is characterized by degenerative changes and death of all cell types, and lasts for a variable period from the second day of culture onwards. Death of the last cells occurs usually between the sixth and ninth days of culture, although in some instances it is delayed for several more days. Cultures differ from each other considerably in the rate and intensity of

* This is a modification of the Wright-Giemsa stain (Michelson and Wilcox, 1940), Leishman being used in the place of Wright powder. For staining, fix air-dried films in the concentrated stain for 1 minute: then dilute stain 1 : 10 with buffered water and leave for 10 minutes.

the various changes, and some of the factors responsible for this variability are by no means clear. Individual tubes of the same culture are, however, similar enough to each other to permit the drawing of a growth curve characteristic for them all.

I. *Growth phase.*

The name of this phase has been chosen, because during the first 36–48 hours of culture there is evidence of proliferation of the immature cells, provided by the presence of cells in mitotic division. From this, it does not follow that there is an increase in the total cell count of the cultures, for there is also a concomitant dying-off of mature cells. If, however, the mitotic index is high enough, as in cultures containing E.E., there may be an increase in the absolute number of immature cells. This means that proliferation outstrips maturation.

Mitotic figures, very rare in the peripheral blood, are regularly found in cultures from the second hour onwards. They occur mainly in myelocytes, but are also seen in myeloblasts. All stages of mitosis are frequently met with. Differential counts have been done as a routine, but the total numbers of mitoses are too small to permit precise conclusions on the phase distribution ; further, owing to the technique necessary in making smears, the recognition of telophases is often difficult, and their number probably greater than is apparent in counts. No gross deviation from the ordinary phase distribution has, however, been found. In particular, there is no evidence of a metaphase arrest in untreated cultures. The mitotic index reaches a maximum of four to six mitoses per 1000 immature cells at about the eighth hour of culture in basic medium, or of up to 19 per 1000 immature cells at about 24 hours, if E.E. is added.

The great majority of mitoses is normal in the first stage of the growth phase. Precise chromosome counts have not been possible, but there has been no single instance of a gross deviation from the diploid number. Minor abnormalities have occasionally been seen. Fig. 11 shows a myelocyte in anaphase, with one certain and another probable chromosome bridge. Fig. 12 shows an early myelocyte anaphase with several " lagging " chromosomes. It is interesting that these abnormalities which are commonly found following the application of X rays occurred in this instance in the absence of treatment. It is, however, usually impossible to decide whether the anaphase bridges are due to abnormal chromosome stickiness or to chromosome interchanges. In the later stages of the growth phase it is common to find pyknotic mitoses. These also occur occasionally in the decay phase.

It is difficult to determine how many immature cells can mature in cultures. Signs of limited maturation can often be observed during the growth phase. Thus, after 8 or 24 hours, one may find myeloblasts with a minimum number of cytoplasmic azurophil granules, a transition stage in the life of the cell at which there is probably no division. One can also often discover collections of normal juvenile polymorphonuclears, another shor -lived stage between immature and fully mature cells. Sometimes it is possible to demonstrate a rise in the absolute number of polymorphonuclears present. There is, however, no proof that a given cell in culture will undergo the complete cycle of normal changes from the myeloblast to the polymorphonuclear, and it is indeed unlikely that it can do so ; but as the growth phase continues, more and more cells show signs of abortive or abnormal maturation.

While there are comparatively few abnormalities of mitotic cells, abnormal " resting " cells can be found even in the earliest stages of culture. Their number increases during the later hours of the growth phase, and becomes very large during the decay phase. All types of myeloid cells are affected ; abnormal immature cells may attempt to ripen, and more mature cells may themselves undergo an atypical development. Either the nucleus or the cytoplasm of a cell may be involved ; and there is frequent asynchronism between the development of these two cellular constituents.

If an 8–24 hour culture is examined, the majority of cells will be normal, but there will also be an increasing proportion of immature cells showing one or more of the following abnormalities:—*Nucleus*: Chromatin pattern of myeloblasts and early myelocytes uniformly dense and coarse, resembling that of early erythroblasts (Fig. 7, 14) ; loss of the nucleoli of the earliest cells (Fig. 3) ; increase in nuclear size (Fig. 2) ; formation of nuclear lobes (Fig. 4, 15). *Cytoplasm*: Appearance and multiplication of vacuoles in myeloblasts and early myelocytes (Fig. 1, 2, 3, 4, 6, 7) ; separation of the cytoplasm of early myelocytes into two layers ; an inner layer surrounding the nucleus and containing granules, and an outer clear basophilic one (Fig. 8, 9) ; appearance of pseudopodia, especially in very early cells (Fig. 2, 3, 4, 5, 8, 9, 10, 16, 17).

At 48 hours the changes are more advanced. The nuclear size has further increased and the lobes can be complex (Fig. 6, 16) ; other nuclei have divided into two parts, each of which is often the size of the parent nucleus (Fig. 5, 17, 18). Metamyelocytes with much convoluted or double nuclei also appear (Fig. 19, 20), and there are a number of completely pyknotic or smeared cells. The overall size of cells may, but need not increase in step with that of their nuclei.

Some changes also take place in the polymorphonuclears. There is an increasing number of multilobed cells, and at an early stage many of them become pyknotic. Early in the culture some juvenile forms appear, as mentioned above, and these seem to be able to age in the normal way.

II. *Decay phase.*

During this phase few mitoses are found, though occasional normal ones have been seen as late as the ninth and tenth days of culture. They cannot, however, influence the downward trend of the growth curve which, in this phase, decreases exponentially with time. The appearance of many of the " resting " cells is now very abnormal, and it is often difficult to classify them (Fig. 10, ? " haemohistioblast "). Monocytoid cells (Fig. 21) are frequently seen ; as these occur in large numbers in cultures which were initially practically free from

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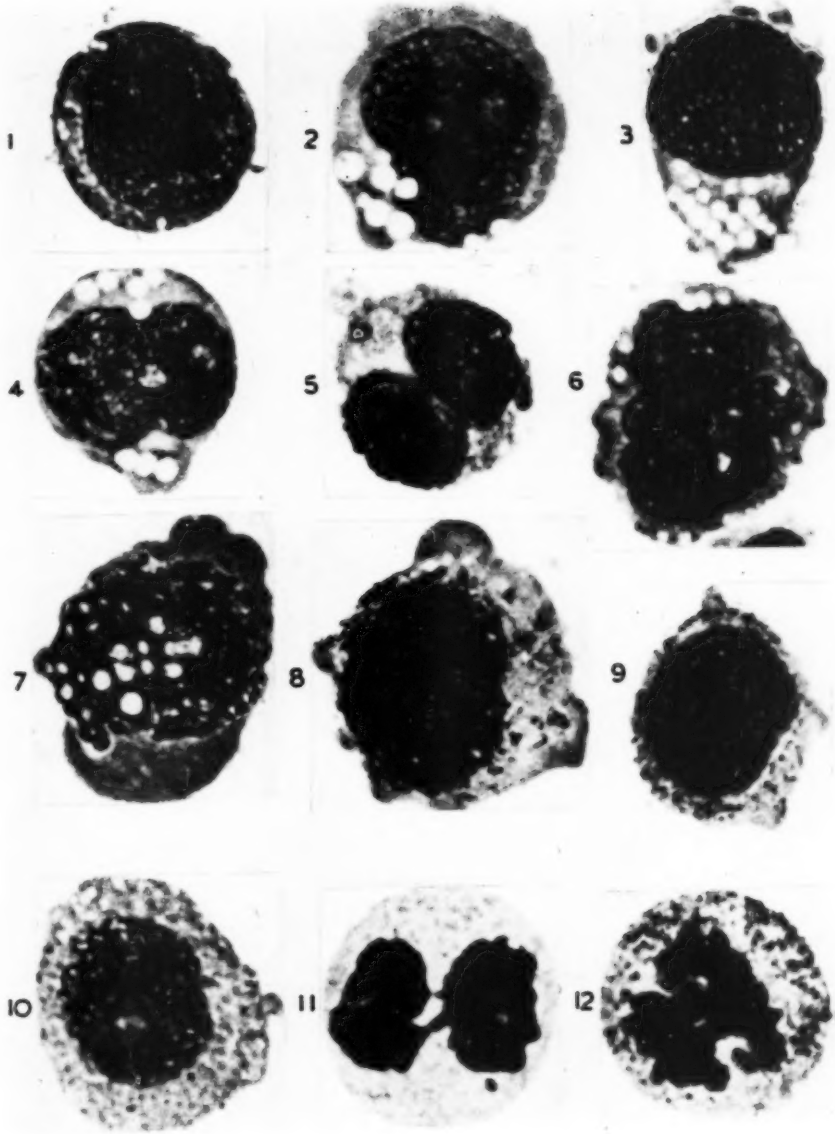
EXPLANATION OF PLATES.

All figures are photomicrographs of abnormal cells from cultures of human blood (chronic myeloid leukaemia), two hours to five days old. The cells were stained in Leishman-Giemsa mixture at pH 6·81–6·85 Magnification × 2130, except Fig. 11, which is × 2600.

FIG. 1–7.—Abnormal myeloblasts from 2–48-hour cultures.
FIG. 8, 9.—Promyelocytes, 48 hours.
FIG. 10.—Unclassified primitive cell, 48 hours.
FIG. 11.—Myelocyte in anaphase of mitosis, showing double chromosome bridge, 24 hours.
FIG. 12.—Myelocyte in early anaphase, 48 hours.
FIG. 13–21.—Myelocytes in various stages of abnormal development, 2 hours–5 days.
FIG. 22, 25.—" Pelger " type of myelocytes, 5 days.
FIG. 23, 24, 26, 27.—" Pelger " type of polymorphonuclears, 48 hours–5 days.



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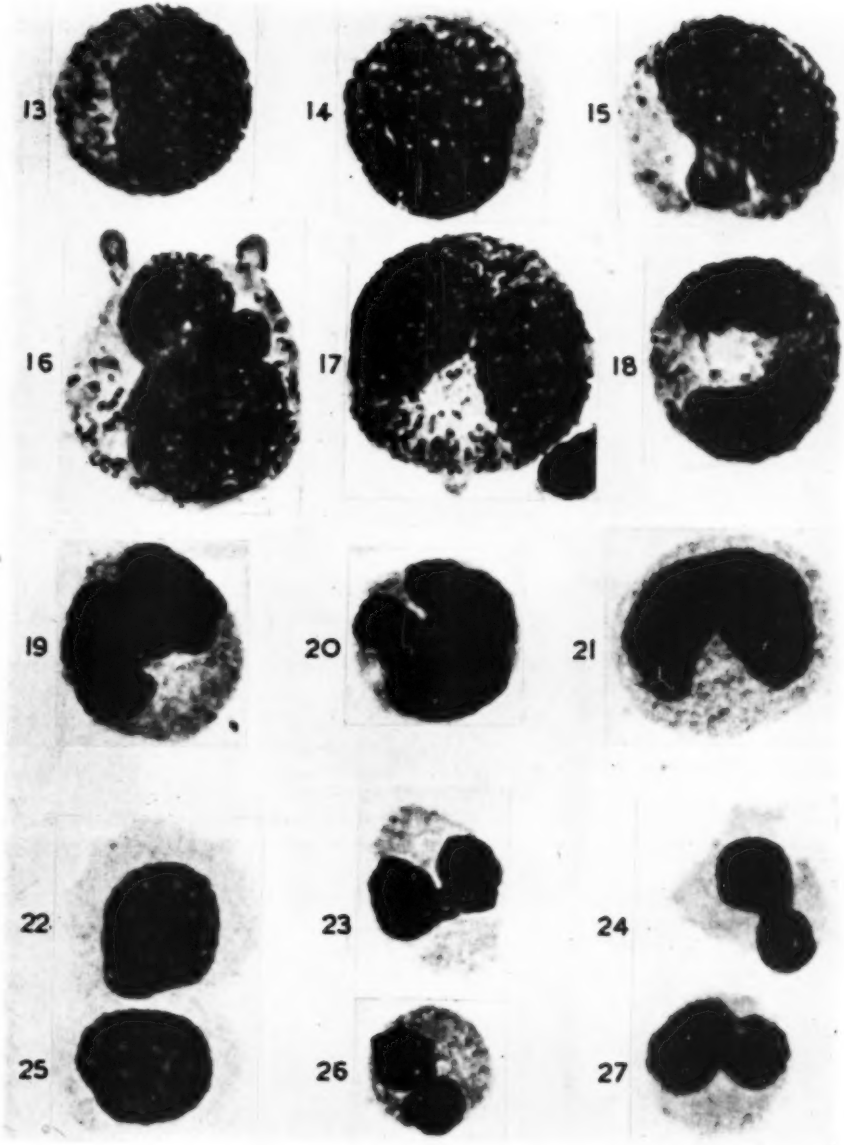
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monocytes, and as transitional stages to myelocytes can be found, it is presumed that they are in fact abnormal members of the myeloid series.

It is rare to find normal mature polymorphonuclears in the later stages of the decay phase. On the other hand, there are often many somewhat unusual forms with uni- and bilobed nuclei. These have a rather denser chromatin pattern than normal cells, and appear to have lost the faculty of developing more complicated nuclei (Fig. 23, 24, 26, 27). They may be derived from certain myelocytes with round dense nuclei (Fig. 22, 25). Both they and their precursors bear a striking resemblance to the cells found in Pelger's anomaly, both in human and animal bloods (Undritz, 1944*a*).

DISCUSSION.

The findings presented in this paper may be compared with those of other authors. An extensive literature deals with tissue culture of normal and leukaemic blood and bone marrow, and has been reviewed by Bloom (1938), and more recently by Fieschi and Astaldi (1946). It is not proposed to discuss this volume of work in detail, especially in view of the fact that much of it is not strictly comparable, as the methods of culturing and of examining the cells were often different from those employed in this series of experiments. Thus most workers have used the cultivation of solid fragments rather than that of cell suspensions, and have described the appearances in sections instead of smears. Comment will therefore be restricted to a few papers in which results have been obtained by related methods.

There is general agreement that survival of human leukaemic cells is possible in cultures, and though the maximum times given vary considerably, no claims of indefinitely prolonged life *in vitro* have been advanced. Many authors report the development of non-specific connective-tissue cell strains such as macrophages and fibroblasts from the highly specialized blood cells, but this appears to depend on the presence of a solid supporting framework, and does not occur in cultures made in a fluid medium.

Mitotic division of immature cells has been frequently observed and generally found normal. It is of interest to note that whereas mitoses are extremely rare in the circulating blood, they begin to appear almost as soon as blood is withdrawn from the body. The rapid change can scarcely be due to the effect of the culture medium alone, but may well be caused by the removal of an inhibitory factor operative in the body.

The occurrence of maturation is a more controversial question, and the various answers which have been given appear to be mostly based on impressions. Strict proof can only be obtained if total as well as differential counts are done with adequate methods, and this is only possible in fluid cultures. Israëls (1940*a*, 1940*b*), working along these lines, maintains that maturation occurs normally. Fieschi and Astaldi, on the strength of smears made from solid cultures, admit a certain degree of maturation, but state that this is always less pronounced than the proliferative processes. The findings cited here and in the previous report tend to support the latter view.

While the normal maturative changes have not been very striking in this series of experiments, the development of new and abnormal cell types has been of interest. It is remarkable that this has not been commented upon by other workers, except by Fieschi and Astaldi, who, in their Fig. 76, show cells very

similar to some pictured here and devote a brief discussion to their significance. They state that such cells do not appear in cultures of normal bone marrow and regard them as peculiar to leukaemic blood—a conclusion which contradicts that of Israëls, who describes as identical the development *in vitro* of normal and leukaemic cells.

In attempting an interpretation of the changes found, it is necessary to postulate a sequence in time. This has been done in Fig. 1–12 and Fig. 13–27. Fig. 1–6 show a series of myeloblasts with progressively severe changes : Fig. 1—normal, apart from several small cytoplasmic vacuoles, Fig. 2—increasing size of the vacuoles, Fig. 3—loss of nucleoli and coarsening of chromatin pattern, Fig. 4—incipient, Fig. 5—complete nuclear division. Fig. 6—progressive nuclear lobulation. Similarly, Fig. 13 shows a normal myelocyte, Fig. 14—coarse chromatin pattern, Fig. 15—early, Fig. 16—advanced nuclear lobulation, Fig. 17—complete nuclear division, Fig. 18—the same in a more mature cell. It is important to stress that such a nuclear division has never been seen to be followed by that of the cytoplasm. The process is therefore not one of amitosis, but more likely an endomitosis. It probably leads, not to reproduction, but to death of the cell.

Endomitosis is known to occur normally in some plants and insects. It has been described by Biesele, Poyner and Painter (1942,) as an important feature of malignant mouse tumours, and by Ludford (1930) as "chromosome formation without spindle development" in several animal tumours. Schwarz (1946) mentions a similar process as occurring in the human marrow in normal and abnormal erythropoiesis. In Fig. 7 and 14 it can be seen that the coarsening of the chromatin pattern is accompanied by its breaking up into discrete fragments, the nuclear membrane remaining meanwhile intact. It is possible that these appearances signify an abnormal prophase, the first stage of endomitosis. The abnormality is conceived to consist in a premature separation of the daughter chromatids, which does not lead to metaphase or to a breakdown of the nuclear membrane. The consequence is an increased nuclear size, which may express itself by lobe formation and can, but need not, lead eventually to a more or less regular nuclear division, unaccompanied by division of the cytoplasm. In this connection the disappearance of the nucleolus is noteworthy : it is apparently a prelude to the accumulation in the nucleus of desoxyribose-nucleic acid, which is made evident by the deeper staining in Leishman-Giemsa and especially in Feulgen-stained films.

It may be objected that small numbers of polyploid cells occur normally in tissue cultures (Macklin, 1916), and that their formation can be encouraged by manipulations such as the reduction of oxygen tension (Barta, 1926) or variations in the temperature of incubation (Stillwell, 1944). Abnormal cells such as are here described might therefore be regarded as insignificant artefacts due to the conditions prevailing in the culture.

To this the reply can be given that though it is impossible to affirm with certainty that the changes observed in cultures are of biological significance, evidence in favour of this view may be seen in the fact that many similar appearances have been found *in vivo*. Cytoplasmic vacuolation has been described in acute leukaemias (Gamberini, 1931) ; immature myeloid cells with convoluted nuclei occur in the marrow of normal foetuses (Levy, 1921), and are frequently observed in the blood and marrow of long-standing cases of chronic myeloid

leukaemia, where their appearance, in large numbers, especially during X ray treatment, presages an early fatal outcome. It is also possible that the large myeloid elements found in the marrow of patients with pernicious anaemia may belong to the same class (La Cour, 1944). Binucleated cells occur in small numbers in normal blood and marrow (Undritz, 1944*b*), and more often in various blood diseases. The curious appearance of the promyelocyte in Fig. 8 with its double layer of cytoplasm has often been described and pictured as typical of the cells of "monocytic" leukaemia (d'Antona, 1931), while the resemblance of some of the polymorphs to those seen in Pelger's anomaly has already been remarked upon.

It may be concluded that immature blood cells can, *in vivo*, undergo changes similar to those which are here reported for *in vitro* work. The special conditions prevailing in cultures made in the fluid medium, and by the technique described, are evidently particularly suitable for encouraging this sequence of abnormal events, but do not permit full normal maturation. It is probable that at least some of the abnormalities in cultures are explained by lack of metabolic factors ordinarily present in the body, and possible that study of the metabolic processes in cultures and of variations in the composition of the medium may elucidate the nature of some of these factors. If successful, such a study might lead to a clearer understanding of the reasons responsible for the varied behaviour in culture of different bloods. As these factors must be inherent in the cells themselves, it is conceivable that when properly analysed, they may give diagnostic or even prognostic importance to tissue cultures of leukaemic blood.

SUMMARY.

The paper gives a description of the appearances in stained smears of human leukaemic blood cells (chronic myeloid leukaemia) when cultured in a fluid medium.

In these conditions, immature cells proliferate by mitotic division and undergo a partial maturation.

There are also formed a series of abnormal cell types, the nature of which is discussed.

I am indebted to the Saltwell Trustees for providing financial support for this investigation. My thanks are also due to Mr. J. A. F. Fozzard, for advice on photomicrography.

REFERENCES.

D'ANTONA, L.—(1931) *Haematologica (Pavia)*, **12**, 527.
BARTA, E.—(1926) *Arch. exp. Zellforsch.*, **2**, 6.
BIESELE, J. J., POYNER, H., AND PAINTER, T. S.—(1942) *Univ. Texas Publ.*, No. 4243.
BLOOM, W.—(1938) *In* Downey's 'Handbook of Hematology,' vol. 2, p. 1469. New York (Hoeber).
FIESCHI, A., AND ASTALDI, G.—(1946) 'La cultura in vitro del midollo osseo.' Pavia (Tipografia del Libro di S. Bianchi).
GAMBERINI, M.—(1931) *Haematologica (Pavia)*, **12**, 511.
GUNZ, F. W.—(1948) *Brit. J. Cancer*, **2**,
ISRAËLS, M. C. G.—(1940*a*) *J. Path. Bact.*, **50**, 145.—(1940*b*) *Ibid.*, **51**, 235.
LA COUR, L. F.—(1944) *Proc. roy. Soc. Edinb.*, B, **62**, 73.

LEVY, F.—(1921) *Z. Anat. Entwicklungsgeschichte*, **61**, 32.
LUDFORD, R. J.—(1930) *9th Sci. Rep. Imp. Cancer Res. Fund., Lond.*, p. 109.
MACKLIN, C. C.—(1916) *Contr. Embryol. Carneg. Instn.*, **4**, 71.
MICHELSON, L., AND WILCOX, A.—(1940) *Publ. Hlth. Rep.*, **55**, 1221.
OSGOOD, E. E., AND BROWNLEE, I. E.—(1936) *J. Amer. med. Ass.*, **107**, 123.—(1937) *Ibid.*, **108**, 1793.
SCHWARZ, E.—(1946) *Amer. J. Anat.*, **79**, 75.
STILLWELL, E. F.—(1944) *Anat. Rec.*, **90**, 115.
UNDRITZ, E.—(1944*a*) *Schweiz. med. Wschr.*, **74**, 995.—(1944*b*) *Folia Haemat.*, **68**, 225.

INCIDENCE OF CARCINOMA IN TRANSMISSIBLE PAPILLOMATOSIS OF THE DOMESTIC RABBIT.

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IN a previous communication we reported that a strain of the Shope rabbit papilloma had been transmitted for 10 serial passages in the domestic rabbit (Selbie and Robinson, 1947). The adaptation of this tumour virus as a transmissible infection in domestic rabbits occurred during experiments in which the simultaneous inoculation of Shope papilloma virus and sheep dermatitis virus in domestic rabbits produced papillomas that proved infective to other rabbits (Selbie, 1946). The further passage of this virus in domestic rabbits was materially helped by continuing the procedure of mixing a second virus suspension with the infective inoculum of papilloma virus, and also by treating the skin with croton oil before inoculation, but there was no evidence of an increase in the virulence of the virus suspensions during passage. In the present communication we shall show that carcinomatous transformation has occurred regularly in this transmissible papillomatosis, and in a manner similar to that found in the non-infective papillomatosis that is produced in domestic rabbits by the inoculation of extracts of papillomas from the cotton-tail rabbit, the original host of the Shope papilloma virus. We shall also discuss the implications arising from the incidence of carcinomatous transformation, and the influence thereon of the ancillary measures that have been used with a view to promoting infectivity.

MATERIALS AND METHODS.

The rabbits to be considered here are those of Passages 2 to 7 which were alive and still carried papillomas 12 months after inoculation, and in addition Rabbit 2 of Passage 9, which has already developed several carcinomas, and died with extensive glandular metastases 40 weeks after inoculation. The early history of the rabbits comprising this series is shown in Table I of the earlier communication, where details are given of the methods used (Selbie and Robin-

son, 1947). In Passages 4 to 9, 4 areas of skin, 2 on either flank, were epilated by plucking out the fur, and 2 areas on the right side were treated with 6 applications on alternate days of 0·3 per cent croton oil in acetone before inoculation. The areas were inoculated following needle scarification with a 10 per cent extract of papillomas, the extract used on the posterior areas containing in addition 10 per cent of glycerolated sheep dermatitis lesions. Thus in all rabbits from Passage 4 in Table I, Area 1 represents normal skin inoculated with a 10 per cent extract of papillomas, Area 2, normal skin inoculated with a mixed extract of papillomas and sheep dermatitis lesions, Area 3, skin pre-treated with croton oil and inoculated with a 10 per cent extract of papillomas, and Area 4, skin pre-treated with croton oil and inoculated with the mixed virus suspensions. Rabbit 2, Passage 2 was inoculated with papilloma extract on normal skin only, and in Rabbit 4, Passage 3, the procedures for Areas 1 and 3 were omitted.

The rabbits were examined at weekly intervals from the 7th week onwards. The tumours were charted and their size was measured by callipers at regular intervals. As was noted in the earlier communication (Selbie and Robinson, 1947), there was a tendency for individual papillomas to unite during development, and in some instances papillomas regressed completely. Hence in this communication only those papillomas that were recognizable as single entities at 6 months will be considered. In areas with confluent or semi-confluent papillomatosis only a few papillomas could be distinguished as independent entities at 6 months, and no accurate count could be made, so that a fair estimate was given of 30 papillomas for confluent papillomatosis and 20 for semi-confluent papillomatosis. It became apparent early in this investigation that if the development of individual papillomas was to be studied over a prolonged period, cancers which arose in adjoining papillomas should be removed. Hence the entire carcinoma was widely excised as early as possible to prevent infiltration of surrounding papillomas (Fig. 3). In practice this was seldom possible to achieve, because at the same time it was necessary to observe the complete development of carcinoma with the formation of metastases.

RESULTS.

Incidence of Carcinomatous Transformation.

Table I shows the incidence of carcinomatous transformation in the individual areas of skin in all the rabbits in these experiments that survived for more than 12 months, and in Rabbit 2, Passage 9, which has already been mentioned. There are still 2 surviving rabbits in Passage 9 which, although they carry papillomas, have not yet, at 40 weeks, developed carcinomas and so have not been included. The carcinomas enumerated in Table I were confirmed histologically after being observed for some time as independent carcinomas arising from single papillomas. The estimate of the number of carcinomas arising on this basis is probably too low, particularly where the original papillomas were confluent or semi-confluent (Fig. 3 and 4). Thus in some instances carcinomatous change has been detected by histological examination in papillomas that appeared normal to the naked eye. Furthermore, it is possible that carcinomas occurring in two adjacent papillomas at approximately the same time would fuse so early as to make their identification as separate individuals unlikely. At present 5 of the 10 rabbits in Table I are alive, and are being kept

TABLE I.—*Number of Carcinomas Developing in Papillomas That Have Survived 6 Months.*

Treatment of inoculated skin and papilloma extract :—
Skin area 1 : No treatment of skin or extract.
" " 2 : Papilloma extract mixed with extract of sheep dermatitis lesions.
" " 3 : Pre-treatment of skin with croton oil.
" " 4 : Treatments 2 and 3 combined.

Passage No.	Rabbit No.	Area number. 1	2	3	4	Period of observation in weeks. Died.	Alive.	Metastases.
2	2	1/5	..	..	..	114	..	Glands and lungs.
4	4	..	0/0	..	2/13	..	103	Glands.
5	1	1/1	1/3	0/9	2/9	91	..	Glands and lungs.
	2	0/30	1/30	0/30	2/30	..	95	..
	3	0/0	1/30	3/8	1/4	77	..	Glands and lungs.
6	1	0/2	0/2	4/20	2/20	..	90	..
	2	0/7	0/8	2/20	2/20	76	..	Glands.
7	1	0/1	0/2	0/0	1/2	..	66	,,
	4	0/4	0/3	0/3	1/2	..	66	..
9	2	2/30	0/30	3/30	1/30	40	..	Glands.
Total	10	4/80	3/108	12/120	14/130	..	..	..

Numerator = Number of carcinomas.
Denominator = Number of papillomas present at 6 months.

for further observation along with rabbits from later passages. All still carry some papillomas with the exception of Rabbit 4, Passage 4, in which the papillomas have all been removed by operation.

It will be seen in Table I that carcinomatous transformation occurred in all the rabbits of Passages 2 to 7 that survived for at least 12 months, and also in one rabbit of Passage 9. It will also be seen that this change was observed in more than one site in 7 of the rabbits, and in as many as six in Rabbit 1, Passage 6 (Fig. 3), and in Rabbit 2, Passage 9, giving a total incidence of 33 carcinomas in the 10 rabbits. There was the expected tendency for more carcinomas to appear in rabbits with more papillomas, but a more significant factor is apparent from the total incidence in all passages of carcinomatous transformation in Areas 1, 2, 3 and 4, as shown in Table I. It will be seen that the sum of carcinomas that occurred in Areas 1 and 3 is similar to that in Areas 2 and 4, so that it is apparent that inoculation of a mixed suspension had no influence on the incidence of carcinomatous transformation. On the other hand, carcinomatous transformation occurred in only 7 of 188 papillomas in Areas 1 and 2 and in as many as 26 of 250 papillomas in Areas 3 and 4, where the skin was treated with croton oil before inoculation. It is thus evident that pre-treatment with croton oil in-

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creased the incidence of carcinomatous transformation in papillomas from about 1 in 27 to about 1 in 9·5, or approximately 3 times. This difference in incidence is just as great if it is based on the papillomas that were counted at 30 to 50 days, shown in Table I of the previous communication (Selbie and Robinson, 1947), in which case carcinomatous transformation occurred in 1 of 20 papillomas on skin pre-treated with croton oil and in 1 of 62 papillomas on untreated skin.

In Fig. 1 are shown the times at which carcinomatous growths were first detected in Passages 2 to 7, Passage 9 being omitted because the surviving rabbits have been observed for only 40 weeks after inoculation. It will be seen that the

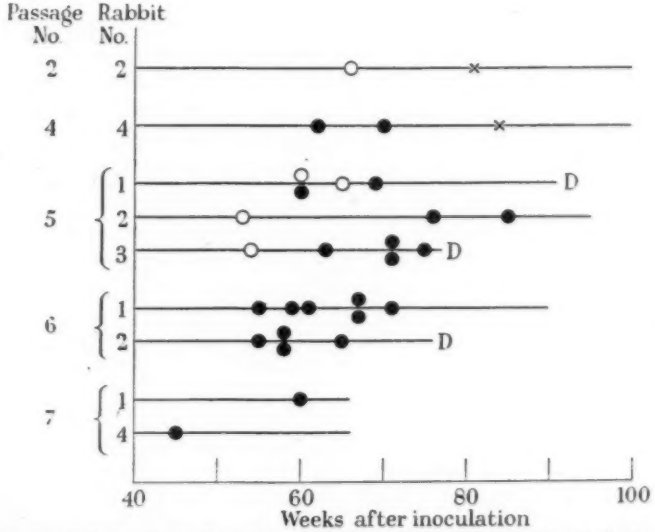


FIG. 1.—Times of observations of carcinomas in all rabbits from the 2nd to the 7th passage. Each rabbit is represented by a line indicating the period of observation from the 40th week onwards. Blocked circles indicate carcinomas arising from papillomas on skin treated with croton oil before inoculation, and open circles indicate carcinomas arising from papillomas on untreated skin.
x= Removal by operation of all remaining papillomas. D = Died.

period before carcinomatous transformation was first observed tended to become shorter with successive passages, while in Rabbit 2 of Passage 9, which is not shown, this period was further reduced to 28 weeks. It is again shown here that the majority of carcinomas occurred in skin pre-treated with croton oil, but it is to be noted that carcinomatous transformation in treated skin was observed over a period of 40 weeks, between 45 and 85 weeks, whereas in normal skin carcinomas appeared only in the relatively short period of 13 weeks between 53 and 66 weeks. There was also an apparent delay in carcinomatous transformation in treated skin, for the mean times of appearance of carcinomas were 65 weeks on treated skin and 60 weeks on untreated skin. That this delay may be of some significance is shown in Passage 5, where there was a later development of carcinomas in treated skin than in untreated skin in all three rabbits. Taking

the results as a whole, however, it can be seen that about half of the total yield of carcinomas on treated skin was obtained after the occurrence of carcinomatous transformation on untreated skin had ceased. Fig. 1 thus shows that treatment of the skin with croton oil not only increased the incidence of carcinomatous transformation, but prolonged the period over which this change was observed.

Incidence of Metastases.

It is shown in Table I that glandular metastases were observed in all the 5 rabbits that have died and in 2 of the 5 rabbits that are still alive. These metastases were found in the lymph nodes of the axilla and groin, and in 3 of the 7 rabbits more than one group of nodes was involved. The time at which glandular metastases were observed varied with the time at which the primary carcinomatous growth occurred, the limits being 33 weeks after inoculation in Rabbit 2, Passage 9, and 89 weeks in Rabbit 2, Passage 2, while the average time was 67 weeks for all the rabbits in the series. Rabbit 1, Passage 6 is interesting in that although 6 different primary cancers occurred between 55 and 71 weeks, no glandular metastases were observed up to the 90th week. It is not possible to draw any definite conclusions from this series concerning the incidence of glandular metastases as many of the primary growths were removed, and in consequence the opportunity for metastases to occur was reduced. Lung metastases were also found in 3 of the 5 rabbits that died. Two died as a result of operative procedures ; in Rabbit 2, Passage 6, there was cirrhosis of the liver with ascites, and Rabbit 2, Passage 9, was emaciated as a result of extensive glandular metastases. Metastases have not yet been observed at any site apart from lungs and lymph nodes.

Development of Carcinoma.

Macroscopical.

In our previous communication the development of the papillomas was fully described, and it was reported that after a period of quiescence or even retrogression, papillomas in some instances finally resumed rapid growth with the manifestations of malignancy (Selbie and Robinson, 1947). The earliest sign of malignant transformation was an increase in the diameter of the papilloma, with a concurrent thickening of the base. In Fig. 2, Area 2, the central papilloma had slowly enlarged from the time of its original appearance to attain a diameter of 1·4 cm. at 53 weeks. Nine weeks later it measured 1·8 cm. in diameter and the base had now thickened and was fleshy in appearance and consistency, in contrast to the dry horny surface shown in Fig. 11. Three weeks later, at 65 weeks, the diameter had increased to 2 cm., and ulceration of the surface had commenced as a result of carcinomatous infiltration. Another early sign of malignant transformation in papillomas was a pink rim of congestion surrounding the base where it merged with normal skin. The rabbits often gnawed these papillomas, presumably because of painful sensations arising from the infiltrating carcinoma, whereas ordinary papillomas rarely caused any sign of resentment. Palpation of such papillomas at this time frequently revealed that the undersurface was convex instead of flat or concave as is usual in quiescent papillomas.

When carcinomatous transformation became visible it usually commenced at the periphery of the papilloma and spread inwards, infiltrating the pigmented

papilloma substance and replacing it with fleshy pink carcinomatous tissue. Simultaneously the invading growth spread under the skin surfaces, causing a typical heaped-up and rolled-edge appearance (Fig. 14). Finally the surface broke down, leaving an ulcer which readily became secondarily infected (Fig. 14). The usual type of carcinomatous transformation was superficial, and gave rise to the fungoid type of growth shown in Fig. 3, Areas 3 and 4, and in Fig. 7 and 10. In other cases, however, carcinomatous infiltration extended under the surface as in Fig. 2, Area 2, where the central papilloma at 62 weeks, although

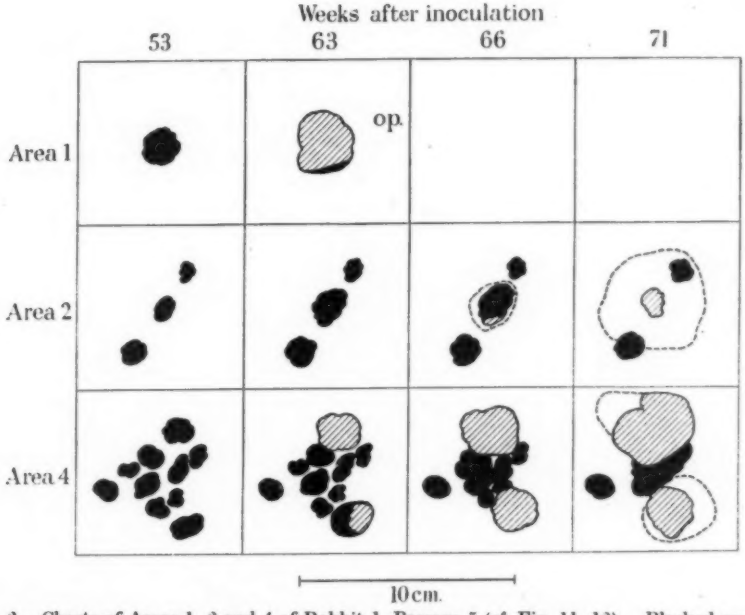


FIG. 2.—Charts of Areas 1, 2 and 4 of Rabbit 1, Passage 5 (*cf.* Fig. 11, 12). Blocked areas indicate papillomas, hatched areas indicate carcinomas, and interrupted lines indicate subcutaneous extension of tumours. *op* = operative removal of carcinoma.

only beginning to show infiltration by carcinoma, was mounted on a mound of carcinomatous tissue (Fig. 11), and 9 weeks later presented the appearance shown in Fig. 12 of a shallow ulcer discharging the contents of keratinized cysts from a large subcutaneous carcinoma. Both types of development of carcinoma are shown in Fig. 4, Area 4 and Fig. 8, 9 and 10, where two tumours showed the first signs of carcinomatous transformation at the 57th week. One of these tumours developed directly into a fungating carcinoma, whereas the other formed a subcutaneous growth which broke through the skin after the 64th week, and finally produced a large fleshy mass similar to the first carcinoma. In many cases superficial carcinomas later showed deep extension in the subcutaneous tissues (Fig. 2, Area 4 ; Fig. 4, Area 3).

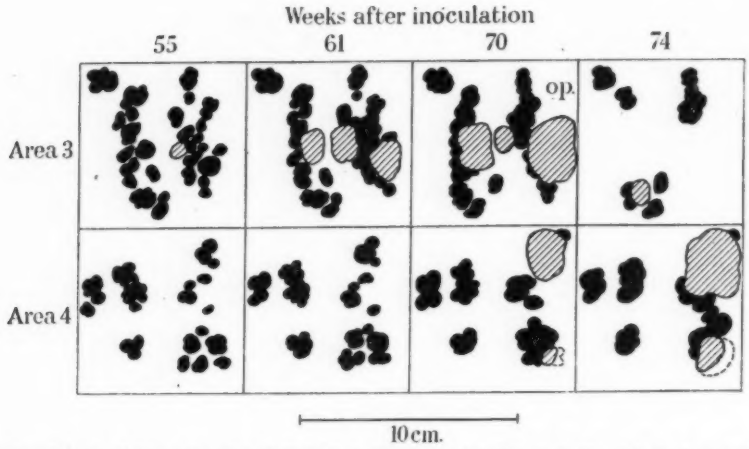


FIG. 3.—Charts of Areas 3 and 4 of Rabbit 1, Passage 6 (*cf.* Fig. 5, 6, 7). Blocked areas indicate papillomas, hatched areas indicate carcinomas, and interrupted lines indicate subcutaneous extension of tumours. *op* = operative removal of carcinoma.

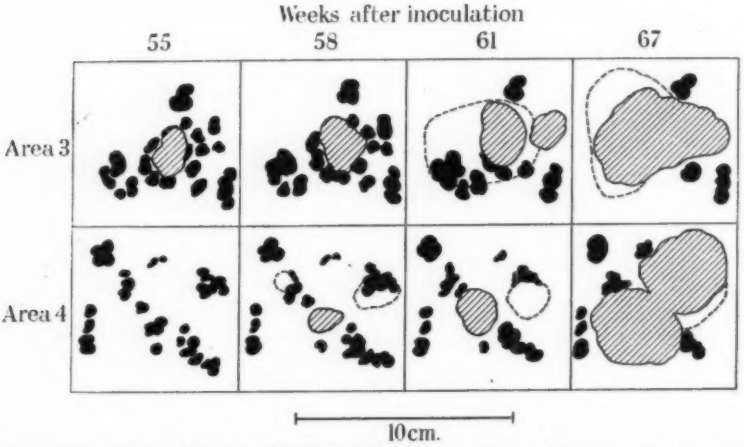


FIG. 4.—Charts of Areas 3 and 4 of Rabbit 2, Passage 6 (*cf.* Fig. 8, 9, 10). Blocked areas indicate papillomas, hatched areas indicate carcinomas, and interrupted lines indicate subcutaneous extension of tumours.

Carcinomas differed not only in their mode of development, as has just been mentioned, but also in their rate of growth. Thus in Fig. 3, Area 3 and in Fig. 5, 6 and 7 it can be seen that the first carcinoma to appear grew slowly and even regressed slightly, whereas the two other carcinomas were more progressive, and of these one grew much more rapidly than the other.

Where lymph nodes became secondarily invaded they usually enlarged rapidly, and were found on section to consist of a thin shell of tissue containing thick, cheesy keratinous material; in some cases these cystic metastases ulcerated to the surface and discharged their contents spontaneously. The metastatic deposits in lung were numerous (Fig. 15), and on section it was found that the larger growths had usually broken down, and formed cysts similar to those in glandular metastases. Fig. 16 shows a huge cyst in the thorax which contained 150 c.c. of caseous material. It arose from a metastasis in the right lung, and almost completely filled the thorax, displacing the heart backwards and to the left. There was also a large cystic secondary deposit in the right axilla.

Microscopical.

All the carcinomas shown in Table I have been examined histologically and have proved to be of the squamous cell type, showing all degrees of differentiation from keratinizing squamous cell carcinoma to anaplastic spindle cell carcinoma (Fig. 17 and 18). In some of the papillomas that have been excised along with malignant growths it has been possible to see early stages of the transformation to malignancy. The first change appears to be an increasing disorganization of the basal epithelial cells, accompanied by increasing numbers of atypical mitoses with incomplete cellular division, leading to the formation of cells of irregular morphology, including giant cells. Following this stage, the malignant process becomes apparent by the breakaway of epithelial cells to form independent islets under the papilloma in the manner described by Rous and Beard (1935).

As the carcinoma develops it either infiltrates the papilloma and subcutaneous tissues and becomes a superficial fungating tumour (Fig. 19) which may become ulcerated (Fig. 20), or it may remain under the papilloma, infiltrating widely and rapidly in the subcutaneous tissue, where the proliferating epithelial cells form strands of carcinomatous tissue (Fig. 21), which ultimately burst through the surface and produce a superficial fungating tumour as in Fig. 19.

The secondary deposits in glands and lungs have all proved to be of the squamous cell type showing varying degrees of differentiation. In many of the lung metastases it can be seen that the infiltrating carcinoma cells form nodules within the alveolar spaces, so that the stroma of the deposit retains the architectural structure of the lung tissue. A small isolated deposit in the lung showing this type of intra-alveolar growth is shown in Fig. 22.

DISCUSSION.

Rous and Beard (1935) first described the carcinomatous change that occurs in Shope rabbit papillomatosis. They found that carcinoma occurred regularly after a period of 9 to 12 months in the uninfective and progressive papillomas of the domestic rabbit, whereas the regressive and infective papillomas of cottontail rabbits had never shown this change. Syverton and Berry (1935) were the first to show that carcinomatous change does occur in cottontail rabbits, and Kidd and Rous (1940) described several carcinomas in cotton-tail rabbits but found that they occurred only on rare occasions. Kidd and Rous (1940) also observed carcinomatous change in papillomas in domestic rabbits that were inoculated with a strain of recoverable virus originally described by Shope (1935).

EXPLANATION OF PLATES.

FIG. 5, 6, 7.—Rabbit 1, Passage 6, Area 3 (*cf.* Fig. 3).

FIG. 5.—58 weeks after inoculation, showing two carcinomas side by side. A papilloma on the right shows a thickened fleshy base. Natural size.

FIG. 6.—62 weeks after inoculation, showing the two carcinomas and a third on the right that has arisen in the fleshy papilloma shown in Fig. 5. Natural size.

FIG. 7.—68 weeks after inoculation, showing considerable increase in size of the third carcinoma and destruction of the surrounding papillomas; of the two other carcinomas one has moderately increased in size and the other shows some regression. Natural size.

FIG. 8, 9, 10.—Rabbit 2, Passage 6, Area 4 (*cf.* Fig. 4).

FIG. 8.—58 weeks after inoculation, showing a central carcinoma, a subcutaneous swelling arising under a papilloma on the right, and a keratin-containing cyst associated with a papilloma on the left. Natural size.

FIG. 9.—61 weeks after inoculation, showing that the central carcinoma has enlarged slightly while the keratin cyst has ruptured and discharged its contents. The subcutaneous tumour is to the right of the carcinoma. Natural size.

FIG. 10.—67 weeks after inoculation, showing considerable enlargement of the original carcinoma and a large fungating carcinoma beside it that has arisen from the subcutaneous tumour. Natural size.

FIG. 11, 12.—Rabbit 1, Passage 5, Area 2 (*cf.* Fig. 2).

FIG. 11.—62 weeks after inoculation, showing the dry horny surface of the central papilloma, from which carcinomatous infiltration has already extended into the underlying tissues. Natural size.

FIG. 12.—71 weeks after inoculation, showing a large subcutaneous tumour with a shallow ulcer which has completely replaced the original papilloma; from the ulcer keratinous material is being discharged. Natural size.

FIG. 13, 14.—Rabbit 1, Passage 5, Area 4 (*cf.* Fig. 2).

FIG. 13.—63 weeks after inoculation, showing above an ulcerated carcinoma, and below the lighter and smoother appearance of part of a papilloma undergoing carcinomatous transformation. Natural size.

FIG. 14.—71 weeks after inoculation, showing two ulcerated carcinomas with rolled edges. Natural size.

FIG. 15.—Rabbit 3, Passage 5, showing numerous metastatic nodules in the lungs. Natural size.

FIG. 16.—Rabbit 1, Passage 5, showing a large cystic metastasis in the right lung displacing the heart to the left. There is a large glandular metastasis in the right axilla. Natural size.

FIG. 17.—Section from Area 4, Rabbit 1, Passage 6 (lower tumour Area 4, Fig. 3) removed 74 weeks after inoculation, showing a typical cell nest in an area of keratinizing squamous cell carcinoma. H. & E. × 300.

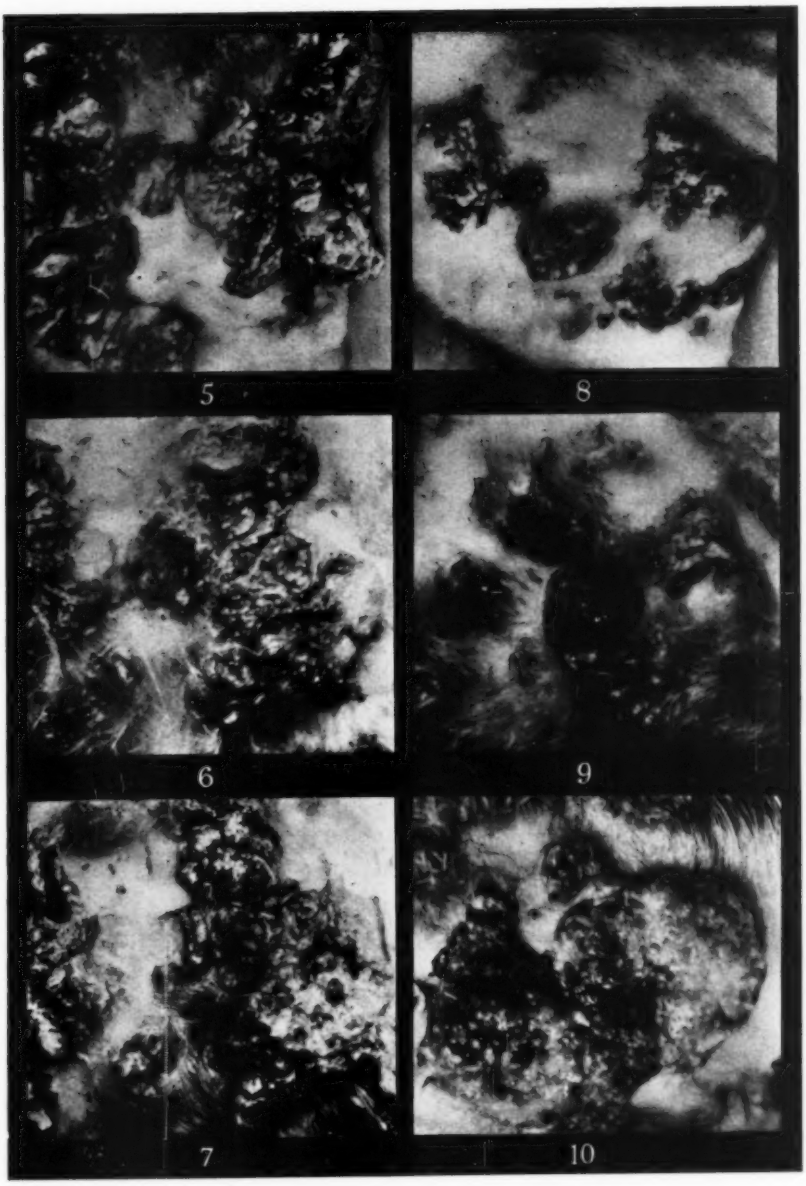
FIG. 18.—Another part of the tumour shown in Fig. 17, showing anaplastic spindle cell carcinoma. H. & E. × 300.

FIG. 19.—Section from Area 4, Rabbit 2, Passage 6 (central carcinoma in Area 4, Fig. 4), 69 weeks after inoculation, showing the mushroom-like appearance of a fungating carcinoma and invasion of the papilloma to the right. H. & E. × 6.

FIG. 20.—Section from Area 2, Rabbit 2, Passage 5, 84 weeks after inoculation, showing a carcinoma that has commenced to ulcerate and subcutaneous infiltration in the rolled edge. H. & E. × 6.

FIG. 21.—Section from Area 2, Rabbit 2, Passage 5, 95 weeks after inoculation, showing a subcutaneous nodule with strands of carcinomatous tissue extending upwards to just under the skin surface. H. & E. × 6.

FIG. 22.—Section of a lung metastasis from Rabbit 2, Passage 2, 114 weeks after inoculation, showing intra-alveolar infiltration. H. & E. × 300.



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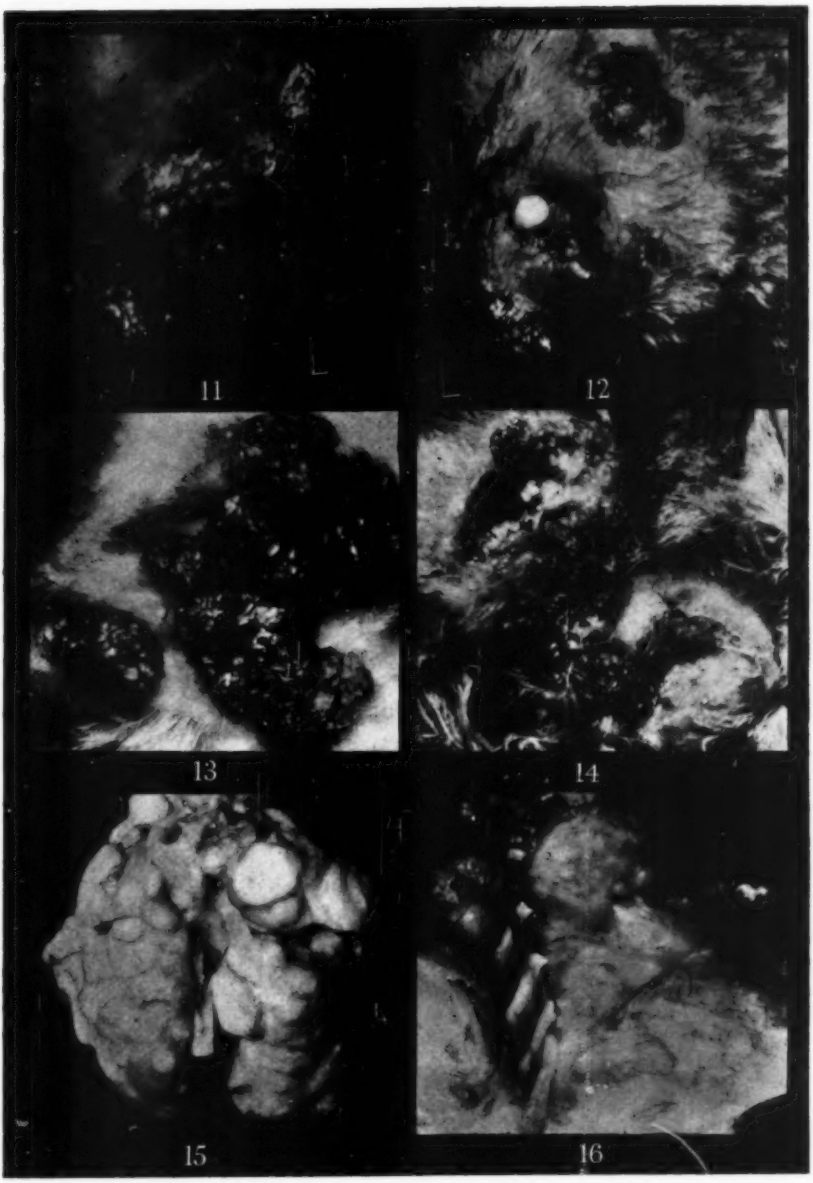

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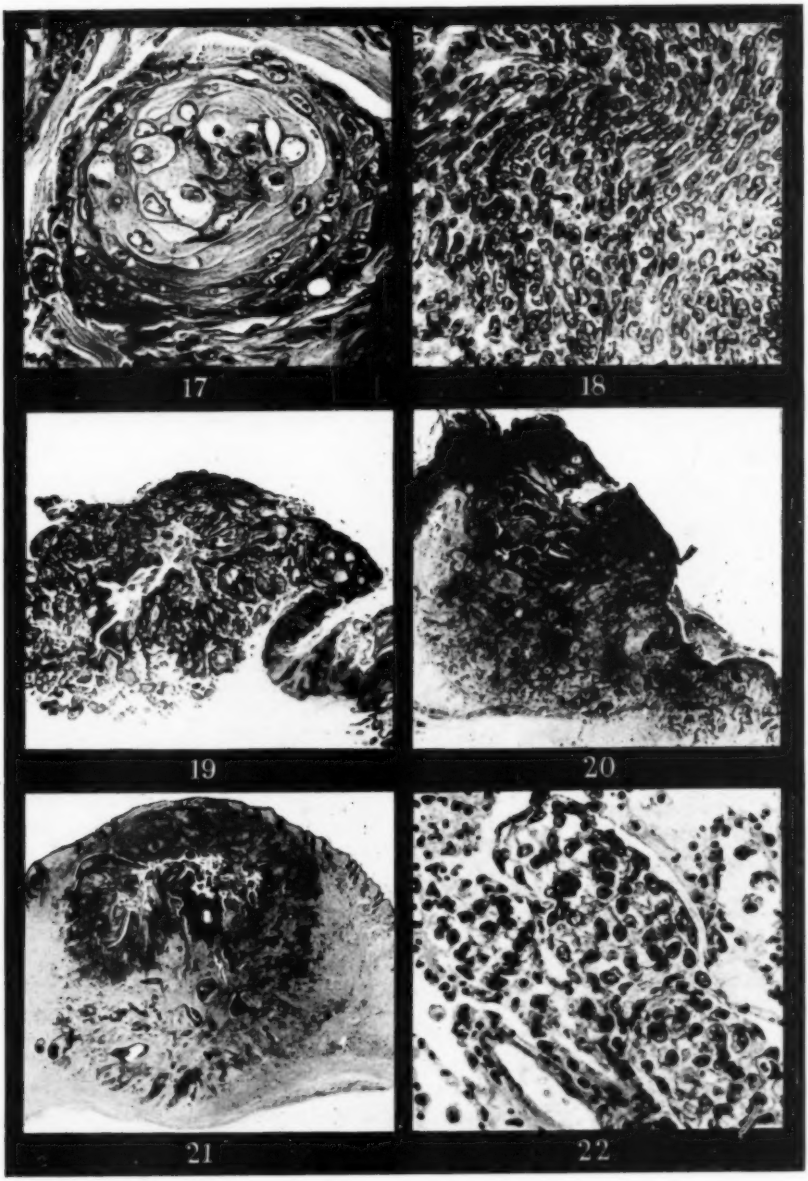

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Selbie and Robinson.

In the experiments described here carcinomatous transformation has occurred regularly in papillomas during serial passage in domestic rabbits of an adapted strain of Shope papilloma virus. This change has been observed in all rabbits bearing papillomas for at least 12 months, and has usually been first noted between the 53rd and 66th week after inoculation. The time at which this change first occurs has tended to become shorter in successive passages, and has been reduced to 45 and 28 weeks in single rabbits of the 7th and 9th passages respectively. This may indicate an increase in the virulence of the virus, but it is more likely to be due to variations in the sensitivity of the rabbits. It has also been shown that the first occurrence of carcinomatous transformation in a rabbit is usually followed by others up to at least 5 in number and up to 85 weeks after inoculation. Indeed carcinomatous transformation occurs so regularly in this transmissible infection that it would appear that all papillomas would show this change, provided that their evolution was not interrupted by the death of the host or by other factors such as spontaneous regression, infiltration from nearby carcinomatous growth, or operative removal. Carcinomatous transformation should then be regarded as a natural stage in the development of infective papillomatosis, and attributable only to the activity of the virus which initiates the infection. It is also clear from the work of Kidd and Rous (1938*a*, 1938*b*) and McIntosh and Selbie (1940), who observed rapid transformation to malignancy in tar-induced papillomas infected with Shope papilloma virus, that this virus is capable of causing immediate carcinomatous change. It is not therefore necessary to postulate an extraneous precipitating factor such as bacterial infection or an alteration in the virus itself to account for the change to malignancy, as suggested by Rous, Beard and Kidd (1936).

Although carcinomatous transformation would appear to be a natural stage in the development of this infection, it presents such a radical change in behaviour that it is difficult to see how it could be part of a continuous process. As has been shown here, indolent papillomas that have remained quiescent for long periods suddenly begin to grow and rapidly assume a fungating ulcerating appearance, and histological changes suggestive of early malignancy are seen only in papillomas in the later stage of their development. It must thus be presumed that the onset of malignant manifestations is due to some relatively sudden alteration in the conditions of growth which may possibly be of a purely mechanical nature, such as pressure from the heaping up of tumour substance which frequently occurs in all advanced papillomas. That pressure may have this effect is indicated by the work of Rous and Beard (1934*a*, 1934*b*), who disturbed the natural growth of Shope papillomas by collodion dressings and by implantation in the tissues and thereby produced tumours, which although they invaded and destroyed tissues surrounding them, did not have the other characteristics of malignant tumours, but caused these effects simply by being confined. In any case it is not likely that the change in the mode of growth of the tumour is due to a new and specific stimulus such as a mutation of the virus, or to the intervention of some carcinogenic agent elaborated during tumour growth, for these stimuli could arise only irregularly, and would not be expected to operate in all rabbits or in numerous papillomas in single rabbits.

An observation that may be of some importance is the higher incidence of carcinomatous transformation in papillomas arising from skin treated with croton oil before inoculation. Since Deelman (1923, 1924) showed that the in-

cidence of tar tumours in mouse skin was increased at the site of injury by deep incision, numerous investigators have demonstrated the enhancing effect of unspecific irritation on the tumour-producing activity of carcinogenic substances. That chemical agents can also have this stimulating effect was shown by Twort and Twort (1928), who accelerated the production of tar tumours in mice by pre-treatment with oleic acid, and by Berenblum (1941*a*, 1941*b*), who obtained a higher proportion of mice with tumours after painting with a dilute solution of benzpyrene by concurrent treatment with croton oil or by applying croton oil after a limited course of benzpyrene treatment. He could not demonstrate any such effect when he treated mouse skin with croton oil for 26 weeks before the application of benzpyrene, but Mottram (1944) in similar experiments with benzpyrene showed that pre-treatment with croton oil for the much shorter period of 3 weeks greatly increased the yield per mouse of both benign and malignant skin tumours.

In virus infections of the skin of the rabbit a response similar to that described by Mottram was observed by Selbie (1946), who found that the pre-treatment of rabbit skin with croton oil described in this communication greatly increased the lesions produced by sheep dermatitis virus, and had some enhancing effect on Shope papilloma virus infection. This procedure has also been of value in maintaining the serial transmission of infectious papillomatosis in the domestic rabbit (Selbie and Robinson, 1947), but it can now be seen from Table I that the overall effect has been an increase in the yield of papillomas by only 1 in 3, whereas the yield of carcinomas has been increased threefold. Croton oil would thus appear to have enhanced the receptivity of the epithelial cells to the virus and so increased the yield of papillomas only to a limited extent, whereas its major role has been to render conditions in the infected cells more suitable for the full activity of this cancer virus and so promote the development of carcinoma.

SUMMARY.

The development of carcinomas from papillomas was observed regularly during serial transmission in domestic rabbits of an adapted strain of Shope papilloma virus.

This was observed in all of 9 rabbits from the 2nd to the 7th passage that survived and carried papillomas for at least 12 months, and also in one rabbit of the 9th passage.

Carcinomatous transformation was observed in more than one papilloma in 7 rabbits, giving a total incidence of 33 carcinomas in the 10 rabbits. The observed incidence of carcinomatous transformation was three times greater in papillomas arising in skin that had been treated with croton oil before inoculation than in papillomas arising in skin that had not been so treated.

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REFERENCES.

BERENBLUM, I.—(1941*a*) *Cancer Res.*, **1**, 44.—(1941*b*) *Ibid.*, **1**, 807.

DEELMAN, H. T.—(1923) *Bull. Ass. franç. Cancer*, **12**, 24.—(1924) *Z. Krebsforsch.*, **21**, 220.

KIDD, J. G., AND ROUS, P.—(1938*a*) *J. exp. Med.*, **67**, 399.—(1938*b*) *Ibid.*, **68**, 529.—(1940) *Ibid.*, **71**, 469.

McIntosh, J., and Selbie, F. R.—(1940) *Rep. Brit. Emp. Cancer Campgn.*, 17, 44.
Mottram, J. C.—(1944) *J. Path. Bact.*, **56**, 391.
Rous, P., and Beard, J. W.—(1934*a*) *J. exp. Med.*, **60**, 701.—(1934*b*) *Ibid.*, **60**, 723.—(1935) *Ibid.*, **62**, 523.
Idem and Kidd, J. G.—(1936) *Ibid.*, **64**, 401.
Selbie, F. R.—(1946) *Brit. J. exp. Path.*, **27**, 143.
Idem and Robinson, R. H. M.—(1947) *Brit. J. Cancer*, **1**, 371.
Shope, R. E.—(1935) *Proc. Soc. exp. Biol., N.Y.*, **32**, 830.
Syverton, J. T., and Berry, G. P.—(1935) *Ibid.*, **33**, 399.
Twort, C. C., and Twort, J. M.—(1928) *J. Hyg., Camb.*, **28**, 219.

OBSERVATIONS ON THE NATURE OF THE OESTROUS CYCLE AND ON THE EFFECT UPON IT OF THE MILK FACTOR, IN MICE OF TWO INBRED STRAINS, DIFFERING IN MAMMARY CANCER INCIDENCE.*

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Allen (1922, 1923) observed great variability in the oestrous cycle in mice of five different strains, and his findings suggested that a genetic factor, which might be linked with coat colour, was a possible cause for some of the variations in cycle length.

Three factors are now known to be concerned in the genesis of mammary cancer in mice, and with the discovery of each, attempts were made to correlate them, and thus mammary cancer incidence, with strain differences in the nature of the oestrous cycle. The first factor to be investigated was genetic constitution. The possibility then had to be considered whether the hereditary condition acted through genetically transmitted differences in ovarian functional activity, or through an unequal response of mammary glands or genital tract to ovarian hormone. Numerous and extensive observations were made with somewhat conflicting results, both on the nature of the oestrous cycle and on variations in organ sensitivity to hormonal stimulation, in strains of mice with differing mammary cancer incidence.

When the existence of the third factor, i.e. the extra-chromosomal milk factor, was established and its importance in the aetiology of mouse mammary cancer was recognized, it was necessary to determine whether this factor acted directly on mammary tissues, or indirectly by means of an effect on cyclical hormonal changes. Support for the latter theory was not forthcoming in the experiments of Van Gulik and Korteweg (1940), or of Deringer, Heston and Andervont (1945).

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The present experiment is an attempt to compare the character of the oestrous cycle, and to demonstrate the effect of the milk factor upon it in two strains of mice differing in mammary cancer incidence.

EXPERIMENTAL PROCEDURE.

The cyclical ovarian changes in four groups of female mice were examined by means of daily vaginal smearing. All the mice belonged to lines inbred by brother-sister mating for many generations.

Group 1.—Twelve mice, possessing natural milk factor, of the RIII strain (Paris), inbred for 18–20 generations. The spontaneous breast cancer incidence observed in recent years is 71 per cent in females living for 6 months or more.

Group 2.—Eleven RIII mice of generations 17–19, deprived of milk factor by foster-nursing on CBA females. The transfer to the foster-mother was made from 0–12 hours after birth.

Group 3.—Ten mice, regarded as free from milk factor, of the CBA strain (Strong), inbred for 53–54 generations. Previous to the 53rd generation no breast cancer had been observed in this strain during 14 years in the laboratory. In litter mates of generation 53, however, two spontaneous mammary cancers were observed at 14 and 16½ months respectively.

Group 4.—Twelve CBA mice of generations 53–55, which received milk factor from RIII foster-mothers at 3–12 hours after birth.

In groups 2 and 4 the period of foster-nursing ranged from 21–31 days.

The mice were kept under identical conditions, in wooden boxes each housing 5 or 6 mice, at constant temperature (65° F.), with daylight lighting only and on a full mixed diet. One mouse from groups 1, 2, and 3 and two from group 4 were each kept in a separate box.

Smears were obtained by the platinum loop method, fixed in absolute alcohol and stained with dilute methylene blue. The criterion accepted as heat was a smear composed of epithelial cells, cornified or nucleated, in which leucocytes were absent, or comprised less than 5 per cent of cells. In the interpretation of the results the statistical significance of any observed difference between averages in the various groups was assessed by the use of Fisher's t factor, probabilities (P) being obtained from his table of t (Fisher, 1944).

RESULTS.

(a) *Age at which vaginal canalization and onset of first oestrus occurred.*

The age at which canalization of the vagina occurred was determined by daily examination of the mice, except where the vaginal canal had been established before weaning (Table I). As the number of determinations was so small, no statistical analysis of the figures was made, but there appears to be no real difference between the strains themselves, nor could the changes produced by foster-nursing be correlated with the presence or absence of the milk factor. As soon as canalization had occurred, or immediately after weaning, daily vaginal smearing was commenced and the onset of the first heat was determined in all cases. This was found to occur at a variable period after canalization, the extreme range for all groups being 6–29 days. There is no statistical difference in the average age of onset of oestrus in the four groups.

TABLE I.—*Age of Onset of Oestrous Cycles in Individual Mice.*

Group of mice.	Interval between birth and transfer to foster-mother in hours.		Period of suckling by foster-mother in days.		Age at canalization in days.				Age at onset of 1st oestrus in days.				Interval between canalization and 1st oestrus in days.			
	2	4	2	4	1	2	3	4	1	2	3	4	1	2	3	4
Mouse No. 1	0	8	22	27	21	—	23	—	35	35	39	51	14	—	16	—
„ 2	8	8	24	31	22	27	23	31	36	36	31	37	14	9	8	6
„ 3	8	11	30	31	21	—	25	37	56	37	37	45	25	—	12	8
„ 4	8	11	30	31	21	—	24	38	34	38	30	47	13	—	6	9
„ 5	8	3	30	29	19	—	24	30	34	36	30	45	15	—	6	15
„ 6	3	8	29	21	28	29	—	28	47	35	44	47	19	6	—	19
„ 7	3	4	29	24	—	29	26	—	37	39	40	47	—	10	16	—
„ 8	3	4	29	24	—	30	26	—	48	37	48	52	—	7	22	—
„ 9	12	12	28	29	—	30	28	—	49	59	45	45	—	29	17	—
„ 10	12	12	28	29	—	28	29	—	39	44	48	49	—	16	19	—
„ 11	12	8	28	23	28	29	—	28	49	48	—	40	21	19	—	12
„ 12	—	8	—	23	—	—	—	28	56	—	—	38	—	—	—	10
Averages	7·0	8·1	27·9	26·8	22·9	28·9	25·3	31·4	43·3 ±2·4	40·4 ±2·2	39·2 ±2·3	45·3 ±1·4	17·3	13·7	13·6	11·3

(b) *The character of the oestrous cycle.*

(i) *Virgin mice.*—Daily vaginal smearing of virgin mice of all groups was continued for periods ranging from 56–137 days immediately following canalization or weaning. The number of complete cycles during the observation period was recorded. In addition, the average length of the whole cycle and of the actual period of heat for each mouse was calculated (Table II). Comparison was then made between the fostered and non-fostered groups (Table III). In all cases comparisons show no significant difference, the P factor being of the order of 0·1–0·5.

A second series of estimations was then made, in which two types of atypical cycle were excluded, in view of the fact that such cycles might have been caused by the repeated smearing technique. In the one type, a prolonged dioestrous interval is observed, a state termed pseudo-pregnaney. This occurs naturally following sterile copulation and artificially following a great variety of stimuli. Parkes (1926) regarded 7 days as the shortest duration of natural pseudo-pregnancy. Therefore all cycles with dioestrus greater than 7 days have been regarded as atypical. In the other type there is prolonged cornification or heat, a condition which has been produced experimentally by repeated vaginal smearing. Parkes (1928) recorded that 96 per cent of heat periods in the unmated mouse lasted from 1–4 days. Thus all those over 4 days were excluded in the revised recordings. The group averages from these second calculations are shown in Table III. Here the difference observed between groups 1 and 2, with regard to the duration of heat, borders on significance ($P = 0{\cdot}01$–$0{\cdot}02$); but in groups 3 and 4 the averages are identical (2·3 days).

(ii) *Parous mice.*—At the end of the first observation period, mice of all groups were mated and 33 out of 44 bore one litter; the remainder bore 2 or 3 litters. The average period of suckling of the 33 single litters was 25 days. Following separation of the litters, daily vaginal examination was re-instituted on the 4 groups of now parous mice and the same calculations made as for virgin mice (Tables II and III).

It will be seen (Table III) that again the only differences in averages to which any statistical significance can be attached are those between the average duration of heat in groups 1 and 2, a result comparable to that observed in the virgin state, when typical cycles only were included in the observations ($P = <0{\cdot}01$).

The averages were then re-assorted and comparisons made between the two strains of mice, whether naturally or foster-nursed (Table III). In normally suckled virgin mice a significant difference was observed between the average duration of the cycle in RIII and CBA groups (1 and 3), when all cycles were included. In both virgin and parous mice there was a difference bordering on significance when atypical cycles were excluded. No such difference was noted between mice of the same strains which had been foster-nursed (groups 2 and 4). The average duration of heat in the two strains showed no significant difference in the naturally nursed groups, and was even identical when atypical cycles were excluded; on comparison of groups 2 and 4, however, P values of $<0{\cdot}01$ and 0·01–0·02 were obtained, in both virgin and parous animals.

(c) *Atypical cycles.*

The pseudo-pregnant state occurred more frequently, and tended to be more prolonged in groups 1 and 2 than in groups 3 and 4; prolonged periods of corni-

TABLE II.—*Duration of Oestrous Cycles and Heat in Individual Mice.*

Group of mice.	No. of days of observation.				No. of oestrous cycles observed.				Average duration of oestrous cycles in days.				Total days of heat during observation period.				Average duration of heat.			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Virgin mice.	75	81	78	66	14	12	13	14	5·4	6·8	6·0	4·7	29	24	33	32	2·1	2·0	2·5	2·3
	74	72	86	81	12	14	19	11	6·2	5·1	4·5	7·4	27	29	51	29	2·3	2·1	2·7	2·6
	56	63	80	79	11	10	13	15	5·1	6·3	6·2	5·3	22	26	40	37	2·0	2·6	3·1	2·5
	77	137	76	63	13	19	13	13	5·9	7·2	5·8	4·8	30	39	33	27	2·3	2·1	2·5	2·1
	63	88	77	78	12	12	14	11	5·3	7·3	5·5	7·1	21	30	38	34	1·8	2·5	2·7	3·1
	95	87	67	75	14	15	12	15	6·8	5·8	5·6	5·0	30	30	29	39	2·1	2·0	2·4	2·6
	100	85	70	63	13	13	14	14	7·7	6·5	5·0	4·5	29	30	34	34	2·2	2·3	2·4	2·4
	89	85	63	60	13	13	13	11	6·8	6·5	4·8	5·5	36	29	27	30	2·8	2·2	2·1	2·7
	80	63	75	80	12	14	16	14	6·7	4·5	4·7	5·7	32	25	39	28	2·7	1·9	2·4	2·0
	97	90	69	62	12	15	14	9	8·1	6·0	4·9	6·9	24	31	35	30	2·0	2·1	2·5	3·3
	93	74	—	70	13	14	—	13	7·2	5·3	—	5·4	30	22	—	37	2·3	1·6	—	2·8
	81	—	—	—	12	—	—	—	6·8	—	—	—	34	—	—	—	2·8	—	—	—
Averages	81·7	84·1	74·1	70·5	12·6	13·7	14·7	12·7	6·5 ±0·273	6·1 ±0·252	5·3 ±0·188	5·7 ±0·303	28·7	28·7	35·9	32·4	2·3 ±0·094	2·1 ±0·084	2·5 ±0·084	2·6 ±0·118
Parous mice.	71	112	67	63	14	15	13	13	5·1	7·5	5·2	4·8	36	26	31	26	2·6	1·7	2·4	2·0
	107	139	85	57	14	17	12	10	7·6	8·2	7·1	5·7	31	32	32	30	2·2	1·9	2·7	3·0
	82	104	62	101	14	13	12	17	5·9	8·0	5·2	5·9	25	25	32	38	1·8	1·9	2·7	2·2
	85	63	47	95	13	9	8	19	6·5	7·0	5·9	5·0	32	20	20	41	2·5	2·2	2·5	2·2
	110	81	75	76	15	12	11	14	7·3	6·8	6·8	5·4	33	28	46	31	2·2	2·3	4·2	2·2
	87	163	91	84	12	21	17	13	7·3	7·8	5·4	6·5	30	48	37	42	2·5	2·3	2·2	3·2
	102	123	73	65	14	14	14	13	7·3	8·8	5·2	5·0	31	28	41	38	2·2	2·0	2·9	2·9
	66	91	86	71	14	14	16	13	4·7	6·5	5·4	5·5	42	25	41	37	3·0	1·8	2·6	2·8
	76	71	68	67	16	13	14	13	4·8	5·5	4·9	5·2	46	27	36	30	2·9	2·1	2·6	2·3
	101	62	69	83	15	14	12	16	6·7	4·4	5·8	5·2	34	30	36	38	2·3	2·1	3·0	2·4
	100	66	127	78	14	15	18	12	7·1	4·4	7·1	6·5	37	32	61	36	2·6	2·1	3·4	3·0
	82	—	—	—	13	—	—	—	6·3	—	—	—	29	—	—	—	2·2	—	—	—
	73	—	—	—	16	—	—	—	4·6	—	—	—	40	—	—	—	2·5	—	—	—
Averages	87·8	97·7	77·3	76·4	14·2	14·3	13·4	13·9	6·2 ±0·309	6·8 ±0·449	5·8 ±0·245	5·5 ±0·175	34·3	29·2	37·5	35·2	2·4 ±0·089	2·0 ±0·06	2·8 ±0·167	2·6 ±0·127

TABLE III.—*Summary of Experimental Results on the Character of the Oestrous Cycle.*

	Group	Averages of duration of all oestrous cycles.	*P* value.	Averages of duration of normal oestrous cycles.	*P* value.	Averages of duration of periods of heat.	*P* value.	Averages of duration of normal periods of heat.	*P* value.
Virgin mice.	1	6·5±0·273	>0·2	5·4±0·159	>0·2	2·3±0·094	>0·1	2·3±0·097	0·01–0·02
	2	6·1±0·252		5·1±0·210		2·1±0·084		2·0±0·063	
	3	5·3±0·188	>0·2	4·7±0·213	>0·7	2·5±0·084	0·5	2·3±0·064	—
	4	5·7±0·303		4·6±0·208		2·6±0·118		2·3±0·099	
Parous mice.	1	6·2±0·309	>0·2	4·8±0·150	—	2·4±0·089	>0·2	2·3±0·068	<0·01
	2	6·8±0·449		4·8±0·169		2·0±0·06		2·0±0·078	
	3	5·8±0·245	>0·3	4·3±0·115	0·5	2·8±0·167	>0·3	2·3±0·093	—
	4	5·5±0·175		4·4±0·093		2·6±0·127		2·3±0·084	
Virgin mice.	1	6·5±0·273	<0·01	5·4±0·159	0·01–0·02	2·3±0·094	>0·1	2·3±0·097	—
	3	5·3±0·188		4·7±0·213		2·5±0·084		2·3±0·064	
	2	6·1±0·252	0·3	5·1±0·210	>0·1	2·1±0·084	0·01	2·0±0·063	0·01–0·02
	4	5·7±0·303		4·6±0·208		2·6±0·118		2·3±0·0995	
Parous mice.	1	6·2±0·309	>0·3	4·8±0·150	0·01–0·02	2·4±0·895	>0·02	2·3±0·068	—
	3	5·8±0·245		4·3±0·115		2·8±0·167		2·3±0·093	
	2	6·8±0·449	0·01–0·02	4·8±0·169	0·05	2·0±0·060	<0·01	2·0±0·078	0·01–0·02
	4	5·5±0·175		4·4±0·093		2·6±0·127		2·3±0·084	

NOTE.—A *P* value of <0·01 is taken as statistically significant; that of 0·01–0·02 as bordering on significance.

fication occurred more often and to a greater extent in the latter two groups than in the former. The incidence and extent of both conditions was unaffected by foster-nursing (Table IV).

TABLE IV.—*Summary of Occurrence and Range of Abnormal Cycles During Observation Periods.*

	Group of mice.	No. of dioestrous intervals of 7 days and over.	No. of mice in which these occurred.	No. of periods of heat of 5 days and over.	No. of mice in which these occurred.	Extreme range of variation in days.	
						Dioestrus.	Heat.
Virgin	1	27	10	3	3	1–15	1–6
	2	21	9	3	3	1–17	1–6
	3	10	7	9	8	1–9	1–9
	4	13	6	11	7	1–12	1–10
Parous	1	32	10	8	6	1–13	1–7
	2	38	10	—	—	1–18	1–4
	3	17	11	14	8	1–10	1–13
	4	15	9	12	7	1–14	1–8

(d) *Age of cessation of oestrus.*

An endeavour was made to determine the age at which regular oestrous cycles ceased and the menopause might be said to have occurred (Table V).

TABLE V.—*Age of Cessation of Oestrus and Mammary Cancer Incidence.*

Group	Age at cessation of oestrus in months.				Observation period after cessation of oestrus in weeks.				Mammary cancer incidence.				Age in months at appearance of mammary cancer.			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
	15+	21	15	18	—	4	4	13	+	+	—	—	14	19½	—	—
	16+	22	15	21+	—	—	8	—	+	—	—	—	14½	—	—	—
	12+	15	8+	18+	—	5	—	—	+	+	—	+	13	16	—	18½
	12+	22	19+	18+	—	4	—	—	+	—	—	+	14	—	—	13½
	15+	22+	19	12+	—	—	5½	—	+	—	—	+	14	—	—	12
	16+	20+	16	15	—	—	9	—	+	—	—	—	16	—	—	—
	15	18	15	12+	13	4	5	—	+	—	—	+	13	—	—	10½
	15+	20+	15	15	—	—	9	8	—	—	—	+	—	—	—	19
	16+	19+	15	15	—	—	19	31	—	—	—	—	—	—	—	—
	14	19+	18	16	4	—	4	18	+	—	—	—	13	—	—	—
	14+	17	12	12	—	5	—	—	+	+	—	—	13	14½	—	—
	16+	—	—	18+	—	—	—	—	—	—	—	+	—	—	—	13
	16+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
									9/13 =69%	3/11 =27%	0/0 = 0%	6/12 =50%				

Figures marked with + represent age at which mouse died, when this occurred before cessation of regular oestrus cycles.

In most mice cycles tend to become extremely irregular before complete cessation. For this purpose each animal was smeared for 7–21 days at ages 9 and 12 months and once a month thereafter, to determine whether regular oestrous cycles were occurring. In some cases death occcurred before the menopause, one mouse dying at 22 months still showing cycles (group 2). The lowest ages at which the menopause occurred in all groups were 14 and 15 months. The death of so many mice while still showing oestrous cycles detracts from the value of the results, but there was some indication that the range of age at which the meno-

pause occurred was later for the RIII groups, but unaffected by foster-nursing apart from the indirect effect of the latter on survival rate.

Further consecutive observations were carried out later, on other suitable old mice of types corresponding to the four groups, to determine whether any clear-cut types of approach to the menopause existed, as well as the age of complete cessation of cycles.

(e) *Mammary cancer incidence.*

Nine mice of group 1 bore mammary cancers, an incidence of 69 per cent (Table V). This is representative of the particular line of RIII mice maintained in the laboratory. Four animals bore multiple tumours.

Three mice of group 2 bore tumours, an incidence of 27 per cent. Delay in transferring the newborn mice to their foster-parents allowed them to receive milk factor from their own mothers, and this was regarded as the cause of the rather high incidence in this group. The interval between birth and transference was less than one hour in the case of one of the 3 mice which later bore tumours; for the other two it was 8 and 12 hours respectively.

The effect of foster-nursing CBA mice on RIII foster-mothers was to raise the mammary cancer incidence from less than 1 per cent to 50 per cent (Table V).

DISCUSSION.

The possibility that the milk factor influences the degree of susceptibility of the genital tract to oestrogens is considered in the experiments of Van Gulik and Korteweg (1940), and Deringer, Heston and Andervont (1945). The former determined the relative sensitivity of the genital tract to artificially administered oestrogens in high and low cancer strains and their reciprocal hybrids. They showed that the low cancer strain was more susceptible than the high, and that both reciprocal hybrids were intermediary in position between their parents. They therefore concluded that the degree of susceptibility bears no relation to the milk factor, but is chromosomally determined. Similarly, Deringer *et al.* (1945) concluded that the difference in age of vaginal canalization, which they observed in two strains of mice and their reciprocal hybrids, was due to genetic factors and not to the maternal extra-chromosomal factor.

In the present experiment, if the milk factor had any influence either on the susceptibility of the genital tract to hormonal stimulation, or directly on hormone production, a clear-cut difference would be expected between the fostered and non-fostered groups of mice, with regard to the character of the oestrous phenomena. Van Gulik and Korteweg suggested that in order to maintain the species, the oestrous cycle will always tend to be normalized by adjustments in hormone production. Thus in contrast with mice in which milk factor has been naturally transmitted for several generations, it might be expected that some detectable difference would be observed before such adjustment could be made, when the milk factor is given artificially to mice not naturally possessing it. Conversely the withdrawal of naturally occurring milk factor might be expected to operate in the reverse way. It was thought that any change induced by these procedures might be most strikingly indicated by the age at which vaginal canalization and the first period of heat occurred. These are the earliest indications of sex hormonal activity, and follow closely on the period of suckling, during which the

milk factor has been administered or withheld. From Table I it is seen that vaginal canalization occurred later in group 3 (free from milk factor) than in group 1 (having natural milk factor), but that the effect of foster-nursing was to increase the age in both cases, so that this cannot be correlated with the administration or withholding of milk factor. The age of onset of oestrus was later in group 1 than in group 3, but although the effect of foster-nursing appeared to influence slightly this age in the expected direction, the observed differences have no statistical significance.

If the relative insensitivity of the genital tract observed by Bonser (1935, Van Gulik and Korteweg (1940) and Mühlbock (1947) in high cancer strains, and therefore in association with the presence of milk factor, were due to the influence of the latter, it is to be expected that this would be revealed in infrequent cycles or shorter periods of oestrus, when the factor was administered artificially. If, on the other hand, increased oestrogen production were the first phenomenon in mammary carcinogenesis and occurred under the influence of the extra-chromosomal factor, even if ordinarily counteracted by decreased sensitivity of the genital tract, then the artificial administration of the factor should cause at first at any rate more frequent cycles, or a higher proportion of the cycles to be occupied by heat. Conversely, artificial withholding of the factor might be expected to operate in the reverse way.

Such cyclical alterations have not been observed, the *P* factor being high in comparisons made between naturally and foster-nursed groups, both with regard to the duration of the oestrous cycle and of heat, with one exception (Table III). This was the duration of heat in virgin and parous mice of groups 1 and 2, when typical cycles only were included. No undue importance has been attached to this as it occurs in the RIII groups, in which foster-nursing is an unsatisfactory procedure, due to the possibility of the offspring receiving milk factor from their mothers during the interval between birth and transference to foster-parent; also the difference is not statisticliay significant when all cycles are included and the samples are small.

On comparing the two strains (Table III) there is some indication that the nature of the cycle shows a strain difference, which is unaffected by foster-nursing. Comparing these results with the features of the oestrous cycle as described by other workers, there are, in general, appreciable strain differences in this respect (Table VI). It may be noted that the average duration of the cycle in young CBA mice as observed by Bonser (1935) is closely comparable with that obtained in the present experiment—5·8 and 5·3 days respectively.

Foster-nursing as a means of administering the milk factor appears to be an entirely satisfactory procedure. Thus the fact that no significant change in the character of the oestrous cycle was induced by this means, would seem to indicate that the factor does not influence the cycle. It is possible, however, that some adjustment to the new conditions created by the foster-mother's milk is made during the period of suckling. The milk factor effect might be counteracted by the time puberty is reached and the hereditary condition alone revealed in the characteristics of the sexual cycle.

The distribution and extent of two forms of atypical cycle were studied, as it was thought that, if they were caused by smearing technique, their incidence might vary in the different groups of mice. Should, however, such cycles occur spontaneously, any observed strain difference in the duration of the cycle or

TABLE VI.—*Comparison of Results Obtained by Various Workers on the Oestrous Cycle in Different Strains of Mice.*

Author.	No. of mice used.	No. of cycles observed.	Coat colour or strain of mice.	Duration of oestrous cycles in days.	Range of cycles in days.	Duration of oestrus in days.	Range of oestrus in days.
Allen (1922)	9	563	Brown	6 M	1–14 D	1–2 A	1–9
			Black, Albino	4 M			
			Yellow, Grey	5 M			
Parkes (1926)	14	56	Albino-unmated	5·7 A	4·3–8·5 A	—	—
(1928)	—	1,000	"Normal-unmated"	6·2 A 5·0 M	2–28	2·4 A	1–11
Zamkof and Rokhlina (1929)	180	581	Albino	5·4 A	1–13 D	2·27 A	1–13
Bonser (1935)	35	256	Strong A young mice	5·7 A	4·1–10·3 A	2·2 A	1·4–3·2 A
	26	209	CBA young mice	5·8 A	4·5–8·7 A	2·4 A	1·8–3·8 A
Deringer *et al.* (1940)	10	—	C_3H-virgin	4·67 A			
	10	—	A „	4·52 A			
Armstrong	12	151	RIII unmated	6·5 A	1–15 D	2·3 A	1–6
	10	141	CBA „	5·3 A	1–9 D	2·5 A	1–9

A = average. M = mode of frequency curve. D = dioestrus.

heat might, as suggested by Allen (1923), be due to them. Such a difference would be reduced or eliminated when typical cycles only were included in the calculations.

Both RIII groups of mice showed more frequent and prolonged pseudo-pregnancies than the CBA groups, but fewer periods of prolonged oestrus (Table IV). It is possible that the genital tract of the high-cancer strain is less sensitive both to oestrin and to the local irritation which tends to cause cornification, a view which would accord with the findings of other workers. On the other hand, the hormonal reaction to a mechanical stimulus simulating copulation may be more sensitive in this strain, and the pseudo-pregnant state initiated more readily. The converse would hold good for the low-cancer CBA strain, in which pseudo-pregnancies are less frequent and prolonged periods of cornification are more numerous. This is of particular interest in view of the results obtained by Bonser (1935), who, using the same CBA strain, showed that there was a somewhat greater reactivity of the vagina to oestrogen on the part of this strain than in Strong A mice.

Cole (1934) described the development of the mammary gland in pseudo-pregnancy as indistinguishable from that during pregnancy, up to 9 days after sterile copulation. It is, therefore, possible that the increased stimulation and development of the mammary gland associated with frequent naturally occurring pseudo-pregnancies may be a factor in the induction of breast tumours in such a strain as the RIII.

Considered both from the point of view of the extent and distribution of the atypical cycles themselves, and from the results of eliminating them from the comparisons of averages (Tables III and IV), it appears that the observed strain differences are due, at any rate in part, to these cycles. There is some indication, however, that apart from atypical cycles there is a fundamental strain difference

in the duration of the cycle, which tends to be longer in the RIII than in the CBA strain.

The present observations on the duration of sexual life and the age at which it ceases (Table V) indicate that these are characteristic for a strain. When the survival age of the RIII mice was increased due to the suppression of mammary tumours by foster-nursing, 6 mice gave an average age for the menopause of 19 months, with others still showing oestrous cycles at 19–22 months. Both CBA groups 3 and 4 gave averages of 16 months in 8 and 4 mice respectively. Later observations also confirmed the delayed occurrence of the menopause in the RIII strain, in which mice at 80–82 weeks were still showing fairly regular cycles. CBA mice showed commencing irregularities at 61 weeks, and complete irregularity at 76 weeks. These observations also showed the great individual cycle variation noted by other workers, during the approach to the menopause. With few exceptions those mice attaining puberty at an early age show a more delayed menopause.

SUMMARY.

A cross-suckling experiment using mice of strains RIII and CBA, which have respectively a high and low spontaneous mammary cancer incidence, has been conducted with the object of observing the effect on the oestrous cycle of administration or deprivation of the maternal milk factor.

Comparisons have been made between fostered and non-fostered groups, and between the two strains, with regard to the following features: the age of puberty, the characteristics of the oestrous cycle, in the virgin and parous state, the incidence and significance of atypical cycles, and the type of approach to and the age of the menopause.

There is some evidence that these features of the sexual life are characteristic for a particular strain and are genetically determined. Foster-nursing appears to have no significant effect upon them.

I am greatly indebted to Dr. Georgiana M. Bonser for her guidance and interest in the planning of this work and for her co-operation in the experimental procedure, which made consecutive daily observations over such a long period possible.

I also wish to thank Dr. J. W. Orr for his advice over the statistical analysis of the experimental results.

REFERENCES.

ALLEN, E.—(1922) *Amer. J. Anat.*, **30**, 297.—(1923) *Ibid.*, **32**, 293.
BONSER, G. M.—(1935) *J. Path. Bact.*, **41**, 217.
COLE, H. A.—(1934) *Proc. Roy. Soc.*, B., **114**, 136.
DERINGER, M. K., HESTON, W. E., AND ANDERVONT, H. B.—(1945) *J. nat. Cancer Inst.*, **5**, 403.
FISHER, R. A.—(1944) 'Statistical Methods for Research Workers,' Ed. 9. London (Oliver & Boyd).
MÜHLBOCK, O.—(1947) *Acta brev. neerl. Physiol.*, **15**, 18.
PARKES, A. S.—(1926) *Proc. roy. Soc.*, B, **100**, 151.—(1928) *Brit. J. exp. Biol.*, **5**, 371.
VAN GULIK, P. J., AND KORTEWEG, R.—(1940) *Amer. J. Cancer*, **38**, 506.
ZAMKOFF, A. A., AND ROKHLINA, M. L.—(1929) *C. R. Soc. Biol., Paris*, **102**, 1003.

BIOLOGICAL TESTS WITH CHOLESTEROL ESTERS OF UNSATURATED ACIDS.

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ALTHOUGH the claims of Roffo (1938, 1939*a*, 1939*b*, 1941) to have induced adenocarcinoma of the stomach in rats given orally fats or cholesterol which had been heated to 350° C. for half-an-hour have not been confirmed (Beck and Peacock, 1941 ; Kirby, 1943, 1944, 1945 ; Morris, Larsen and Lippincott, 1943), the positive results obtained by other routes in mice by other workers make a good case for regarding the products of such pyrolyses as carcinogenic. Thus Beck (1941) found sarcomas in 2 of 12 mice at the site of injection of cotton-seed oil, previously heated to 340–360° C. for 1 hour, and Steiner, Steele and Koch (1943) reported sarcomas in 3 of 9 mice injected with sesame oil heated to 350° C. These latter workers found no tumours in similar mice injected with cholesterol that had been heated to 200° C. or to 300° C., for 2 hours, but Beck, Kirby and Peacock (1945) observed a spindle-cell sarcoma in 1 of 8 mice surviving more than a year after subcutaneous injection with cholesterol that had been heated to 300° C. for half-an-hour, and another sarcoma in 1 of 9 mice surviving more than 340 days after subcutaneous injection with a residue left after removing dicholesteryl ether and Δ4cholestenone from cholesterol heated in the same way ; these tumours were closely associated with the sites of injection. Negative results were obtained by these workers when mice were injected subcutaneously with cholesterol that had been heated to 430° C. for half-an-hour, or with the product of heating cholesteryl stearate or palmitate to 300° C. for half-an-hour ; these materials were tested for carcinogenicity by the oral route in rats by Kirby (1945), who reported entirely negative findings.

The possibility still remained that, although the common esters of cholesterol with saturated fatty acids, namely, the stearate and palmitate, do not readily yield carcinogenic residues on pyrolysis at normal maximum cooking temperatures, the esters with naturally-occurring unsaturated fatty acids, such as the oleate, which is a normal constituent of blood (Walker, 1930), and linoleate, might do so. It was therefore planned to examine the effects of feeding cholesteryl oleate and linoleate, heated to 300° C., to rats, and also to test these products by painting and subcutaneous injection in mice. Unfortunately, it was not possible to prepare enough of either ester for the feeding experiments, as the war-time supply failed first of oxalyl chloride and, later, even of phosphorus pentachloride. However, the base-line experiment with commercial cholesteryl oleate was carried out and is recorded below. Sufficient of both esters was prepared for the tests in mice by both routes.

EXPERIMENTAL.

(a) *Commercial cholesteryl oleate in rats.*

The material used for this experiment was the ordinary product marketed by British Drug Houses, Ltd., and was used without purification. The makers kindly informed us that only cholesterol and oleic acid were used in its manufacture, and that the temperature required did not exceed 150° C.; pyrolysis would therefore be very slight.

Fifteen Wistar rats, bred in this Department, 8 males and 7 females, were maintained on a diet of rat-cake (Thomson, 1936), plus occasional greenstuff, in such quantity that they were always hungry each morning. 25 mg. of cholesteryl oleate, impregnated in a piece of rat-cake by dropping on it 0·1 ml. of a 25 per cent solution in chloroform and allowing the solvent to evaporate, were fed every morning to each rat, which ate its share at once. The feeding of the oleate was continued until the rat died or until 615 days, the surviving animals being then maintained on untreated rat-cake. The last survivors were killed 2 years after the feeding of the oleate had commenced.

Results.—Only 1 rat failed to survive a year's experimentation, and this male died after 349 days; there were no significant lesions. Of the remaining 14, 5 died or were killed between 400 and 500 days, 1 was killed at 533 days, 1 died and 2 were killed between 500 and 600 days and 3 were killed at 735 days. Bronchiectatic abscesses were found in most animals surviving 400 days; there was no other frequent lesion. The stomachs were all normal, save for that of a rat dying after 616 days which had the haemorrhagic erosions of the glandular zone commonly found in rats in a state of inanition. The only other lesion of interest was in one of the rats killed at the end of the two-year period; this was found to have an extensive, highly-differentiated, infected adenocarcinoma of the uterus, presumably of spontaneous origin.

(b) *Cholesteryl oleate (heated) in mice.*

Good yields (60–70 per cent) of oleyl chloride were obtained by refluxing oleic acid, freed from saturated acids, with oxalyl chloride (Daubert, Fricke and Longenecker, 1943). Cholesterol, purified *via* the acetate and recrystallized *ex* petroleum ether, 60–80° C., until non-fluorescent, was heated on the water-bath for 2 to 3 hours with a slight excess of oleyl chloride (Christiani, 1938). The crude oleate was dissolved in chloroform, washed with N sodium carbonate solution, transferred to petroleum ether (60–80° C.) solution and poured through a tower of alumina (B.D.H.). The filtrate was concentrated and the residue crystallized from ethanol. White, non-fluorescent, cholesteryl oleate was obtained, m.p. 40° C. (turbid), clear by 60° C.

The pure oleate, obtained without pyrolysis, was heated in an open beaker to 300° C. for 2 hours. It was found that 64 per cent of the first batch of oleate was converted to volatile products after half-an-hour's heating; after 2 hours only 16 per cent of tarry material remained. This residue $(C.O.)_H$ was not entirely soluble in acetone, but was soluble in benzene, which was used as the solvent for painting experiments. For subcutaneous injection the residue was dissolved in arachis oil.

Painting experiment.—16 male and 13 female mice of mixed colours were used ; the benzene solution (10 per cent $C.O._H$) was applied thrice weekly to the nape of the neck after the hair had been clipped away. Shortly after the paintings had been commenced, 0·5 per cent of croton oil was added to the benzene solution in the hope of speeding up or enhancing the action of any carcinogen present in the $C.O._H$. After 258 days' experimentation, a 15 per cent benzene solution of $C.O._H$, containing 0·5 per cent croton oil, replaced the original 10 per cent solution ; 2 males and 5 females survived long enough to be treated with the stronger solution. Three other male mice were painted with the 10 per cent solution for 168 days and then with the 15 per cent solution.

Atrophic changes at the site of painting were seen in mice dying at 80 days after the beginning of the experiment, but even in 2 females painted 258 days with the 10 per cent solution and then 374 days with the 15 per cent solution, and surviving totals of 640 and 656 days respectively, no further lesion was found. Three mice survived 400 to 500 days and 4 for 500 to 600. Fourteen mice showed fatty changes in the liver, sometimes with extensive necrosis, but no hyperplastic changes were seen in this organ. No other frequent lesion was found in these mice.

Injection experiment.—12 male and 11 female mice of the same stock were injected subcutaneously in the right flank with 0·5 ml. arachis oil, containing 20 per cent $C.O._H$. After 27 days another injection was made in the left flank with 0·5 ml. of a 24 per cent solution in arachis oil. The first mouse to die survived 80 days after the first injection ; 2 others died before 100 days, 10 survived 100 to 200 days, 2 from 200 to 300 days, 2 from 300 to 400 days, 5 from 400 to 500 days, and 1 survived 518 days.

A mild histiocytic reaction was seen around sites of injection, but no sign of any tumour was ever found. Fatty changes in the liver were rare, but in mice dying at 126, 130 and 384 days after the first injection there were leukemic changes involving the liver and spleen in the first, the liver in the second, and the liver, spleen, kidney and lungs in the third mouse.

(c) *Cholesteryl linoleate (heated) in mice.*

Linoleic acid was prepared from cottonseed oil (*Organic Syntheses*, 1942) and converted to the acid chloride by refluxing with oxalyl chloride. Purified cholesterol was heated with linoleyl chloride on the water-bath for 3 hours, and purified as for the oleate. The pure linoleate was heated in an open beaker to 300° C. for 2 hours. For painting, a 10 per cent solution of the residue ($C.L._H$) was prepared in benzene containing 0·5 per cent croton oil ; a 25 per cent solution in arachis oil was used for injection.

Painting experiment.—10 male and 9 female mice of mixed colours were painted thrice weekly on the nape of the neck after the hair had been clipped away. Painting was continued until 593 days had elapsed ; 3 males and 2 females survived this period, 1 dying at 596 days and the remainder being sacrificed at 640 days.

Atrophic changes at the site of painting were seen in most mice, including the first to die (80 days) ; only ulcer was found apart from this reaction. Four mice showed changes in the liver, spleen and kidney which appeared to be amyloid in nature. One female was killed after 302 days because of a large, solid mammary

tumour in the left flank near the thigh ; another, killed at 640 days, had a cystic adenocarcinoma in the right axillary region.

Injection experiment.—11 male and 9 female mice of the same stock were injected subcutaneously with 0·5 ml. of the oily solution in the right flank. One female died after 8 days ; the rest received another injection of 0·5 ml. in the left flank, 31 days after the injection in the other flank. Eight mice survived 100 to 200 days after the first injection, 1 for 247 days, 1 for 357 days, 2 for 470 days, 1 for 509 days, 1 for 558 days and 1 for 653 days.

At the site of injection nothing beyond a mild histiocytic reaction was found in any mouse. The mouse killed at 509 days had a mammary adenocarcinoma in the right flank. Necrosis of the liver and spleen were seen in 10 mice ; 4 mice showed definite leukaemic infiltration.

DISCUSSION.

These experiments constituted an attempt to determine whether a carcinogenic residue would be left when unsaturated-fatty-acid esters of cholesterol were heated to a temperature not much above those attained in ordinary cooking in ovens, for a period not grossly exceeding that required for this purpose. The fatty acids selected were oleic (1 double-bond) and linoleic (2 double-bonds) ; both occur naturally in fats, and cholesteryl oleate is known (Walker, 1930) to be a natural constituent of blood and therefore of meat. The temperature selected was 300° C. and the time was 2 hours.

As the esters were prepared by a non-pyrolytic method and were subsequently purified till non-fluorescent, any polycyclic hydrocarbon present in the residue after heating would have arisen as a direct result of the heating. No chemical experiments have been made to determine the nature of the components of the residue. But the biological tests reported here gave no evidence of carcinogenicity in these residues for the skin or subcutaneous tissues of stock mice. Such tumours as did arise in the mice used were almost certainly spontaneous. Damage to the liver and the spleen was frequent, and the occurrence of leukemic infiltration was significant and probably attributable to the injections.

It now seems that cholesterol and its naturally occurring esters may not be an important source of carcinogens in heated food. The results of the experiments described in this paper lend no support to the results reported by Beck, Kirby and Peacock (1945), who administered cholesterol heated to 300° C. for half-an-hour. In view of the absence of carcinogenicity of cholesterol heated to 430° C. for half-an-hour and of cholesterol stearate and palmitate heated to 300° C. for the same period, as recorded by those authors, also of the negative findings of Steiner, Steele and Koch (1943) with cholesterol heated to 200° and to 300° C. for 2 hours, and of the negative results with cholesterol oleate and linoleate, heated to 300° C. for 2 hours, reported in this paper, the carcinogenicity of such heated cholesterol or its esters would seem to be very slight.

SUMMARY.

1. No tumours have been induced in Wistar rats given orally cholesteryl oleate (commercial) for up to 615 days and allowed to live up to 2 years.

2. No tumours have been induced in stock mice painted with benzene

solutions, containing croton oil, of the residues of either cholesteryl oleate or cholesteryl linoleate which had been heated to 300° C. for 2 hours.

3. No tumours have been induced in stock mice injected subcutaneously with arachis oil solutions of the aforesaid residues.

4. It is concluded from this work and from other reports in the literature that cholesterol and its esters heated to reasonable cooking temperatures for a reasonable cooking period have not been proved to yield carcinogenic residues.

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REFERENCES.

Beck, S.—(1941) *Brit. J. exp. Path.*, **22**, 299.
Idem, Kirby, A. H. M., and Peacock, P. R.—(1945) *Cancer Res.*, **5**, 135.
Idem and Peacock, P. R.—(1941) *Brit. med. J.*, i, 81.
Christiani, A. F. v.—(1938) *Z. Krebsforsch.*, **48**, 366.
Daubert, B. F., Fricke, H. H., and Longenecker, H. E.—(1943) *J. Amer. chem. Soc.*, **65**, 2142.
Kirby, A. H. M.—(1943) *Cancer Res.*, **3**, 519.—(1944) *Ibid.*, **4**, 94.—(1945) *Ibid.*, **5**, 129.
Morris, H. P., Larsen, D. C., and Lippincott, S. W.—(1943) *J. nat. Cancer Inst.*, **4**, 285.
Organic Syntheses (1942), **22**, 75. New York (Wiley).
Roffo, A. H.—(1938) *Bol. Inst. Cancer*, B. Aires, **15**, 837.—(1939*a*) *Bull. Ass. franç. Cancer*, **28**, 556.—(1939*b*) *Z. Krebsforsch.*, **49**, 341.—(1941) *Bol. Inst. Cancer*, B. Aires, **18**, 929.
Steiner, P. E., Steele, R., and Koch, F. C.—(1943) *Cancer Res.*, **3**, 100.
Thomson, W.—(1936) *J. Hyg., Camb.*, **36**, 24.
Walker, E.—(1930) *Biochem. J.*, **24**, 1489.

FACTORS DETERMINING THE ACTION OF COLCHICINE ON TUMOUR GROWTH.

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In a review devoted to the discussion of colchicine as a possible chemotherapeutic agent for cancer (Ludford, 1945), attention was directed to the dual action of this drug on tumours. It is the most potent of mitotic poisons, and as the action of these substances is greatest where cell division is most frequent, colchicine has a relatively greater effect upon rapidly growing neoplasms than upon most tissues of adult organisms. Furthermore, since endothelial cells of growing capillaries are particularly sensitive to its toxic action, it induces extensive haemorrhage in the soft highly cellular rapidly growing types of tumours. Evidence was adduced that regression of malignant growth produced by colchicine is mainly the result of this vascular damage. Considerable variation in the response of different tumours to treatment with this drug has been reported, and the present paper represents an attempt to elucidate the factors which are responsible for such differences.

The "colchicinic effect."

An experiment with a carcino-sarcoma of Strong *A* mice will serve to demonstrate the typical reaction to colchicine of a soft, highly cellular and rapidly growing tumour in a strain of mice the growing capillaries of which are highly sensitive to its toxic action. Some account of this carcino-sarcoma has already been given in a previous paper (Ludford and Barlow, 1945). At the time the experiment to be described was performed, sections exhibited large compact groups of carcinoma cells, surrounded by an abundant and highly cellular stroma, composed of enlarged and hyperchromatinic spindle-shaped and polymorphic cells, presenting the typical cytological appearance of sarcoma cells. This tumour was transplanted by subcutaneous grafting into the right axillae of ten young adult Strong *A* mice. Seven days later colchicine was injected subcutaneously into 5 mice. An aqueous solution was employed in a concentration of 1 part in 10,000 of distilled water. This had been prepared six days previously, when it was maintained at the temperature of boiling water for ten minutes to reduce the possibility of infection, and was stored in the dark for use throughout the experiment. Since the activity of colchicine is reduced by high temperatures and by keeping, especially when exposed to light, the solution employed for this experiment was rather less potent than a freshly prepared solution. On the seventh day injections were made as follows :

1st injection	.	0·1 c.c. dorsally.
2nd ,,	.	0·2 c.c. ventrally—30 min. later.
3rd ,,	.	0·3 c.c. dorsally—2½ hr. later.
4th ,,	.	0·1 c.c. ventrally—1 hr. later.

Altogether 0·7 c.c. of fluid containing 0·07 mg. of the alkaloid over a period of four hours.

The following day all the mice so treated were ill. They were kept covered with cotton-wool at room temperature. With one exception all recovered. The very definite effect which this treatment had upon the subsequent growth of the tumours is seen in Fig. 1 by comparing the size of the tumours of the controls and the treated mice on the 21st day.

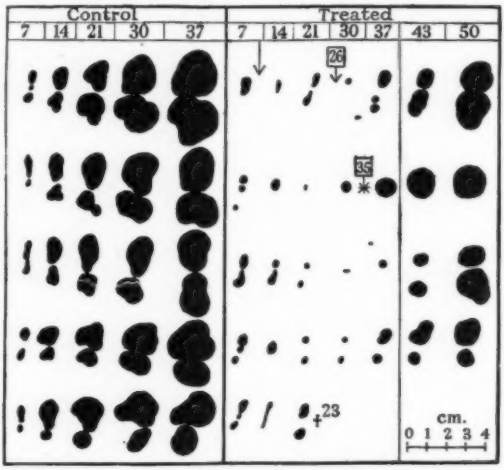

FIG. 1.—Action of colchicine upon a carcino-sarcoma (D) in Strong *A* strain mice. Figures indicate days after transplantation, arrows denote treatment, and crosses death of mice in all figures. In this experiment one mouse only was treated on the 35th day.

By the 26th day the four surviving treated mice had recovered and were re-injected as follows :

1st injection	.	0·1 c.c. dorsally.
2nd ,,	.	0·2 c.c. ventrally—45 min. later.
3rd ,,	.	0·2 c.c. dorsally—2 hr. later.
4th ,,	.	0·1 c.c. ventrally—2 hr. later.

Altogether 0·6 c.c. of fluid containing 0·06 mg. of the alkaloid, over a period of $4\frac{3}{4}$ hours.

Again the mice suffered from the toxic action of the drug, but survived. When the tumours were charted four days later, on the 30th day, the difference between those of the treated and control mice was considerable (Fig. 1). The tumour of one of the treated mice seemed to have regressed completely. No nodule was perceptible on palpation. However, as shown in the chart, all the tumours subsequently recurred and grew progressively. One mouse received a third series of injections on the 35th day as follows :

1st injection	.	0·4 c.c. dorsally.
2nd ,,	.	0·2 c.c. ventrally—4 hr. later.
3rd ,,	.	0·1 c.c. dorsally—2 hr. later.

Nevertheless malignant growth was progressive.

It will be observed from the chart (Fig. 1) that by the 50th day after transplantation the tumours of the treated mice had attained approximately the same size as the controls reached on the 30th day.

In other experiments with the same tumour there has been occasionally complete regression of growth. This occurs when the treatment is repeated

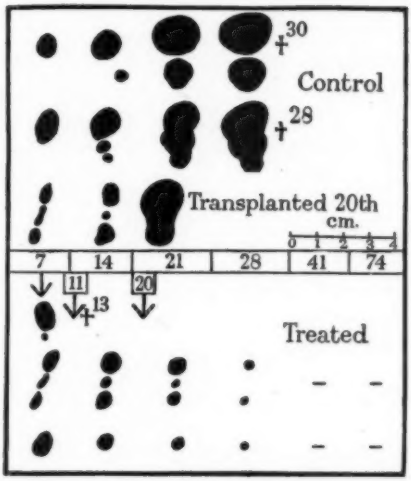

FIG. 2.—Regression of a sarcoma (C) in Strong *A* strain mice after treatment with colchicine.

after the shortest possible interval consistent with survival of the mice. Such treatment is invariably accompanied by a high mortality.

An experiment with a spindle-celled sarcoma in Strong *A* mice demonstrates the possibility of completely inhibiting malignant growth. This sarcoma was one which originated by the sarcomatous transformation of the stroma of a mammary carcinoma. Mice into which it is transplanted do not usually survive long, as it rapidly penetrates the skin and leads to considerable superficial ulceration. Of six young adults transplanted with this sarcoma three were treated 7, 11 and 20 days after transplantation. On each occasion they received a single injection of 0·048 mg. of colchicine. This was freshly prepared for the first injection, 8·0 mg. of the drug being dissolved in 50 c.c. of distilled water and kept in boiling water for 4 minutes. The chart (Fig. 2) shows that two of the treated tumours regressed completely and the mice recovered. On the 74th day after transplantation they were killed and examined for traces of sarcoma tissue. None was found.

It should be emphasized that complete inhibition of malignant growth by colchicine is exceptional. What may be considered the typical "colchicinic effect" is regression of growth followed by recurrence. The magnitude of the effect induced by the same dose of the drug varies very considerably with different tumours, and also with the same type of tumour in different strains of mice.

The factors which have been found to determine the extent of the " colchicinic effect " may be classified as follows :

Host factors	1. Age.
	2. Genetic constitution.
	3. Cumulative toxic action.
Tumour factors	4. Rate of growth.
	5. Histological structure.
	6. Acquirement of tolerance.

Factors influencing the " colchicinic effect."

1. *Age.*—Since actively growing tissues are most sensitive to mitotic poisoning, it is to be expected that young animals would be more susceptible than older ones to the toxic action of the drug. Ries (1939) reported that a 42-days-old mouse will survive more than 17 times the dose of colchicine which is lethal to one 10 days old.

The maximum inhibition of malignant growth occurs with the highest tolerated doses of the drug. Therefore, to obtain the greatest arrest of tumour growth it is necessary to employ animals at the age when they are most resistant to the general toxic action and the largest doses can be administered. The best results have been obtained with young adult mice. Old mice are less able to tolerate the drug. Thus, 11 Strong *A* mice varying in age from 18 months to 2 years and 12 young adults were each injected with 0·056 mg. of colchicine. Within 3 days five of the old mice died and the remainder were almost moribund, but all the young adults survived.

2. *Genetic constitution.*—Experiments have been carried out with tumour-bearing mice of four inbred strains and various hybrids. The mice of the inbred strains fall into three groups according to their sensitivity to the toxic action of the drug, thus :

Group 1 . .	R III.
„ 2 . .	C 3H.
„ 3 . .	Strong *A* and C 57.

The largest single doses which it has been found practicable to employ in the treatment of similar types of tumours in these strains are in the ratio of 5:4:3. But while R III mice will tolerate doses which would be lethal to *A* or C 57 mice, a quantity of the drug which induces considerable regression of growth in an *A* mammary carcinoma has a negligible effect on a mammary carcinoma growing at the same rate in R III mice. Furthermore, the extent of the haemorrhage induced in the same tumour growing in mice of different strains varies, although the mice may be equally sensitive to the toxic action of the drug. Mice of the *A* and C 57 strains employed tolerated the same single large doses of the drug, although the *A* mice survived repeated doses rather better. It has been observed that when tumours of a similar histological type and rate of growth (both sarcomas and carcinomas) in these strains of mice are treated with the same doses of colchicine, the tumours of the *A* mice become more haemorrhagic and regress more than those of the C 57 strain. A transplantable spindle-celled sarcoma originally induced by Dr. Dmochowski in C 57 mice by methyl-cholanthrene has been transplanted into both C 57 and *A* strain mice. A single injection of 0·048 mg.

of colchicine induced greater regression of tumour growth in the *A* strain. The difference in the reaction of another tumour growing in these two strains is demonstrated by the following experiment:

Sarcoma 37 S which has been transplanted for many years in mixed hybrid mice was transplanted into mice of the *A* and C 57 strains. Mice of approximately the same weight were selected for this experiment. One half of those of each strain served as controls; the others were injected with colchicine on the 7th and

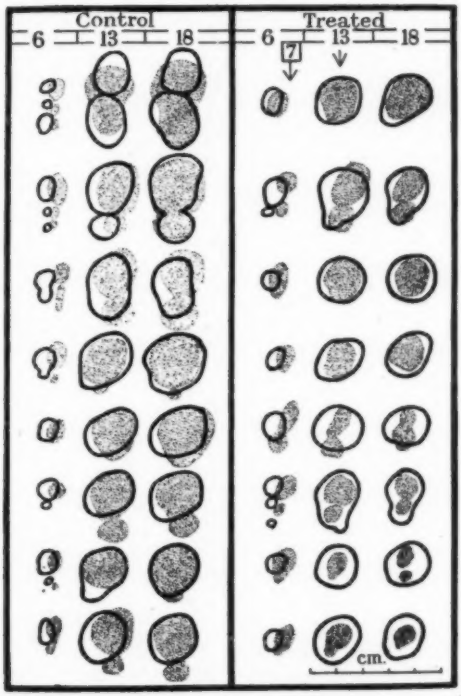

FIG. 3.—Action of colchicine upon sarcoma 37 grown in Strong *A* and C 57 strain mice. Tumours in C 57 mice indicated by outline, tumours in *A* mice stippled. Tumour regression is greatest in the *A* mice.

13th days after transplantation. At each injection each mouse received subcutaneously on the back 0·032 mg. of colchicine in 0·2 c.c. of distilled water. Fig. 3 records the size of the sarcomas when charted on the 6th, 13th and 18th days after transplantation. The size of the tumours in the C 57 mice is indicated in outline, and in the *A* mice by the stippled areas. It will be observed that there is little difference in the size of the tumours in the control animals. Actually growth is slightly more rapid in the *A* mice, but there is a definite difference in the two strains of treated mice. Tumour growth in *A* mice is inhibited more than in C 57 mice. Twenty-four hours after the first injection one mouse of each

strain of those treated (not included in the chart) was killed for examination of the tumour. That in the *A* mouse exhibited considerably more haemorrhage.

Attention has been directed by several investigators to the similarity in the action on tumours of colchicine and bacterial filtrates, and the relevant literature was discussed in a recent review (Ludford, 1945). It is of interest to recall that Andervont (1936) demonstrated differences in the susceptibility to *B. coli* filtrates of sarcoma 37 when grown in different inbred strains of mice, and also " in different lots of stock mice " obtained from dealers.

3. *Cumulative toxic effect.*—It is impossible to administer repeatedly large doses of the drug to animals owing to its cumulative toxic effect. After several injections at short intervals within the dosage range necessary to induce tumour regression, mice lose weight and become almost completely depleted of fat tissue. Attempts have been made to find an antidote to colchicine poisoning, but without success; saline and glucose solutions, tissue extracts, atropine, adrenaline, desoxycorticosterone acetate and thiol compounds have been injected into mice suffering from colchicine poisoning, but without beneficial results.

4. *Rate of tumour growth.*—Tumours of various types in different inbred strains of mice have been transplanted for comparative study. The magnitude of the " colchicinic effect " with tumours of any one inbred strain is, in general, proportional to their rate of growth. Below is summarized the result of experiments with mice of four inbred strains. The dosage of colchicine is that administered to young adults of each strain. The figures in brackets, after each tumour, indicate the approximate rate of growth in comparison with that of the *A* carcinosarcoma (Fig. 1). The tumours of each strain are arranged according to the extent to which they undergo regression following treatment. Thus it will be seen that the action of colchicine upon slow-growing tumours is negligible.

TABLE I.

Extent of "colchicinic effect."	Inbred strain:— A	C 57	C3H	R III
	Dosage:— 0·048 mg.	0·048 mg.	0·064 mg.	0·08 mg.
+++	Carcino-sarcoma D (1)	..	..	Mammary carcinomas D and E (1).
	Sarcoma C (1)			
++	..	Sarcoma D (>1)	Mammary carcinoma B (2/3)	Sarcoma B (2/3)
				Mammary carcinoma C (2/3)
	..	Mammary carcinomas B and C (2/5)	..	..
+	..	Squamous-cell carcinoma (2/3)	..	..
	Lung carcinomas B (1/8) C (1/10)	Histiocytoma (1/10)	..	Fibro-sarcoma (2/3)

5. *Histological structure.*—Two tumours in Table I (the C 57 squamous-celled carcinoma and the R III fibro-sarcoma) responded less to the drug than would have been anticipated on the basis of their rate of growth. Both were characterized by their relatively firm consistency. It seems probable that in tumours of this type the blood capillaries are not so readily disrupted. This aspect of the problem, however, requires further investigation. The application of the transparent-chamber technique devised by Algire (1943) might reasonably be expected to extend our knowledge of the action of drugs upon different types of tumour capillary systems.

6. *Acquirement of tolerance.*—After tumour-bearing mice have received several large doses of colchicine further treatment is less successful in bringing about tumour regression. This is illustrated by the growth of the carcinoma in the sixth of the treated mice in the experiment described below (Fig. 4). A similar acquirement of tolerance by tumours to the haemorrhage-inducing action of bacterial polysaccharides has been reported by Shear (1944).

Application of the preceding results for obtaining the maximum "colchicinic effect."

Discrepancies in the reports on the action of colchicine on neoplasms which have been published are explicable on the basis of the preceding findings. Furthermore, they indicate the experimental conditions under which the maximal effect on tumour growth is most likely to be obtained. The following experiment was planned so as to establish the most favourable conditions for obtaining tumour regression.

Carcinoma 63 was selected as being a soft, highly cellular tumour with large numbers of mitotic figures. It was transplanted into R III mice because of the strains available they tolerate the largest doses of the drug, and their newly-formed capillaries undergo considerable destruction following the administration of large doses. Therefore, by this procedure the carcinoma cells were subjected to the highest possible concentration compatible with survival of the mice, so that the maximum direct toxic action on the malignant cells was combined with extensive vascular damage. Further, since the carcinoma cells were not homozygous with the cells of the mice into which they were transplanted, an additional factor favouring the experimental inhibition of tumour growth was introduced.

In assessing the significance of the results of any experiments of this kind it is necessary to take into account the results of grafting carcinoma 63 in R III mice. These have been as follows :

227 mice of the R III strain transplanted with carcinoma 63.

192	—tumours grew progressively . . .	84·6 per cent
15	—no growth	6·6 ,,
4	—regressed within two weeks . . .	1·8 ,,
16	—regressed after two weeks . . .	7·0 ,,

In Fig. 4 is recorded by weekly chartings the growth of carcinoma 63 in the control and treated mice. In the case of the eight controls only five chartings are included. The figures at the top of the diagram give the number of days after transplantation when the tumours were recorded. It will be observed that all but one grew progressively. One mouse was killed on the 21st day,

and its tumour was grown in tissue cultures in order to test its growth rate *in vitro*. It was found to be normal for this carcinoma. The experimental mice were treated on the 14th day after transplantation with a 1 in 10,000 solution

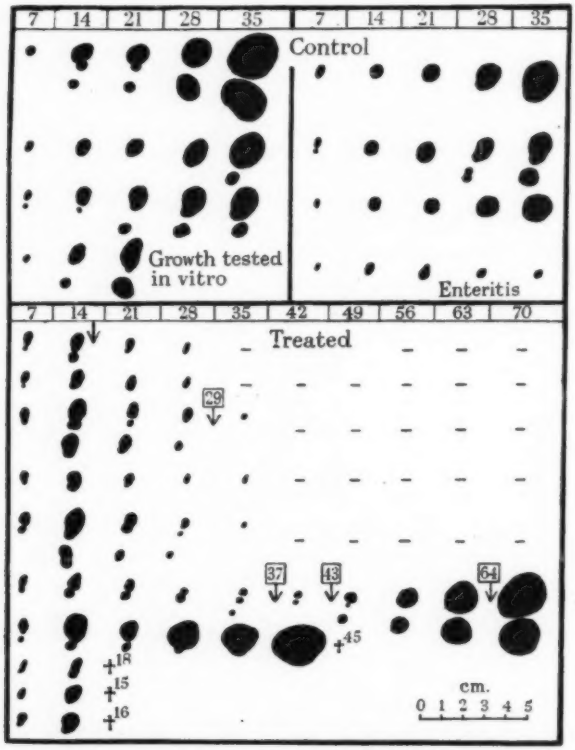

FIG. 4.—Action of colchicine upon carcinoma 63 grown in R III strain mice, which tolerate the largest doses of the alkaloid.

of colchicine in distilled water which had been maintained at the temperature of boiling water for ten minutes. The treatment was as follows :

1st injection . 0·4 c.c. dorsally.
2nd ,, . 0·3 c.c. ventrally—3 hours later.
3rd ,, . 0·2 c.c. dorsally—2 hours later.

Altogether 0·9 c.c. of fluid containing 0·09 mg. of colchicine over a period of five hours.

As is usual with such large doses the mice suffered severely. Three succumbed, one each on the 15th, 16th and 18th days. Of the survivors, the tumours of two

regressed completely, and the remainder were treated again on the 29th day as follows :

1st injection . 0·3 c.c. dorsally.
2nd ,, . 0·3 c.c. ventrally—2½ hours later.
3rd ,, . 0·2 c.c. dorsally—2 hours later.
Altogether 0·8 c.c. of fluid containing 0·08 mg. of colchicine over a period of 4½ hours.

Two other tumours regressed completely, but the remaining two continued to grow progressively. Both received the same treatment on the 37th day. One died with a large tumour on the 45th day. The other received two subsequent treatments to which it failed to respond. The five mice which had been completely " cured " were kept under observation for a year and were then killed. Post-mortem examination revealed no trace of carcinomas.

The charting of all the tumours in these experiments was carried out independently by Mr. A. Chapman, the Senior Technician in charge of this work.

DISCUSSION.

With all the tumours which underwent regression in the preceding experiments there was extensive haemorrhage following treatment. This was most readily induced in the *A* strain mice. A few hours after the injection of the maximum sub-lethal doses of the drug the tumours appeared blue through the skin. Subsequently superficial ulceration was of common occurrence.

Since Boyland and Boyland (1937) first drew attention to the similarity of action of colchicine and bacterial filtrates they have been shown to behave alike in most other respects. With both, complete regression of some transplantable and spontaneous tumours has been effected. The results acquire significance in that they indicate the limitation of haemorrhage-inducing agents in the treatment of malignant growths.

That some form of damage to the capillary system of tumours might be the best way to inhibit malignant growth was suggested by Woglom (1922). A critical review of the evidence concerning tumour regression led him to raise the question whether " the receding tumor may not differ from the growing one only in the extent to which its blood vessels have been obliterated by thrombosis." He pointed out that the blood vessels of many tumours are more subject to thrombosis than those of normal tissues, and he conceived the possibility of both chemical and mechanical factors being responsible. The former resulted from the abnormal metabolism of malignant cells, and the latter was described as " the mechanical pressure which the rapidly increasing parenchyma must often exert upon its vessels, with all the possibilities of injury to their walls which this would entail."

More recently Algire (1945) has devised an adaptation of the transparent chamber technique which enables microscopic observations to be made of the development of the blood vessels of tumour transplants. He reports that although many vessels in growing tumours become very large, " differentiation into arterioles and venules was not evident." In mammary cancers he observed the development of " blood-filled cul-de-sacs." " These appeared to result from

occlusion of the tumour capillaries by pressure of the growing cells." It was suggested that rupture of these " cul-de-sacs " was responsible for the haemorrhagic character of mammary carcinomas.

Mechanical pressure of tumour parenchyma upon capillaries was also cited by Mottram (1928) as the cause of vascular obstruction in tumours following irradiation. He pointed out, and the writer confirmed (Ludford, 1932), that malignant cells which had been irradiated may undergo considerable hypertrophy during the period of mitotic arrest. This enlargement of the malignant cells by imposing pressure upon the sinusoidal-like capillaries was considered to result in their obliteration, and in stagnation thrombosis.

A similar mechanical process might contribute to the vascular damage caused by bacterial filtrates and colchicine. Apitz (1933) first reported that cancer cells acquired an oedematous appearance after treatment with bacterial filtrate, and Andervont (1936) remarked that " careful examination reveals swelling of some tumor cells prior to the hemorrhage, indicating that the bacterial products may have some direct effect upon the tumor cell." He observed that the blood vessels became dilated before rupturing. Gerber and Bernheim (1938) as the result of their histological studies came, however, to the conclusion that the effect of filtrate on tumours was the result of a direct action upon malignant cells rather than upon the capillaries.

The direct action of colchicine upon cell division needs no reiteration. It is, however, pertinent to point out in this connection that the cells arrested in mitosis become considerably swollen, so that Barber and Callan (1943) described their condition as a state of " intracellular dropsy." As very large numbers of cells of rapidly growing tumours exhibit this condition after treatment with the alkaloid, increased pressure of the tumour parenchyma upon the capillaries would appear inevitable. This, and to a much greater extent the toxic action of the drug upon newly formed capillaries, would seem to contribute towards a reasonable, if not complete, explanation of the haemorrhage-inducing action of colchicine.

While emphasizing the major role of haemorrhage in bringing about tumour regression, the possibility of a direct toxic action of the alkaloid on tumours has not been ignored. Experimental work with a wide range of animal tumours demonstrates that there is considerable variation in the sensitivity of malignant cells to toxic substances both *in vivo* and *in vitro*. There would also seem to be differences in the sensitivity of cells of the same tumour to mitotic poisons, as it is not uncommon in tissue cultures of tumours treated with colchicine, and in the tumours of animals which have been injected with the drug, to find amongst numerous arrested metaphases a few cells which appear to be completing mitosis in the normal manner. It is most unwise to generalize about tumours from experiments with a few selected types, and the possibility should not be overlooked that with some new growths the direct toxic action on the malignant cells may play a much greater part in bringing about tumour regression. This might be expected to be the case with lymphomas and lymphosarcomas, since thymocytes and lymphocytes are usually the first cells to exhibit injury when toxic substances are injected into animals (Dustin, 1934). With the tumours used in the present investigation, however, the intratumoral haemorrhage was so extensive as to render it unlikely that the direct toxic action on the cancer cells was a significant factor in contributing to the retrogressive process.

In discussing the significance of haemorrhage upon tumour growth Andervont and Shimkin (1939) wrote: "Our experience has indicated repeatedly that the regression of transplanted tumors after bacterial filtrate treatment is present only when a gross hemorrhage is produced in the tumor, and that regression is directly proportional to the amount of hemorrhage. Thus, if only the central portion of the tumor becomes hemorrhagic only that area regresses, while the growth of the non-hemorrhagic periphery is uninterrupted." Exactly the same statement would apply to colchicine treatment.

SUMMARY.

1. Treatment of soft, highly-cellular, rapidly growing mouse tumours with colchicine, in dosages just below the lethal dose induces haemorrhage, which results in tumour regression. Typically there follows recurrence of growth (Fig. 1), but occasionally complete regression is obtained (Fig. 2 and 4).

2. Factors which influence the "colchicinic effect" are:

A. Host factors.

(i) *Age.*—Young adults tolerate the biggest doses and enable the greatest regression of tumour growth to be effected.

(ii) *Genetic constitution.*—This determines (*a*) the maximum dosage tolerated, and (*b*) the sensitivity of the tumour capillaries to the destructive action of the drug (Fig. 3).

(iii) *Cumulative toxic action.*—This limits the repeated application of the drug at short intervals.

B. Tumour factors.

(i) *Rate of growth.*—The degree of response to treatment is, in general, proportional to the rate of tumour growth in any one inbred strain (Table I).

(ii) *Histological structure.*—Tumours which respond best to treatment are the soft, highly-cellular type.

(iii) *Acquirement of tolerance.*—After several large doses tumours respond less to treatment (Fig. 4).

3. Attention is directed to the similarity in the action of bacterial filtrates and colchicine upon tumour growth. With both, tumour regression is primarily the result of haemorrhage, and the degree of regression is dependent upon the extent of the haemorrhage.

REFERENCES.

ALGIRE, G. H.—(1943) *J. nat. Cancer Inst.*, **4**, 1.—(1945) *Ibid.*, **6**, 73.
ANDERVONT, H. B.—(1936) *Amer. J. Cancer*, **27**, 77.
Idem AND SHIMKIN, M. B.—(1939) *Ibid.*, **36**, 451.
APITZ, K.—(1933) *Z. Krebsforsch.*, **40**, 50.
BARBER, H. N., AND CALLAN, H. G.—(1943) *Proc. Roy. Soc.*, B, **131**, 258.
BOYLAND, E., AND BOYLAND, M. E.—(1937) *Biochem. J.*, **31**, 454.

DUSTIN, A. P.—(1934) *Cancer*, Brux., **11**, 1.
GERBER, I. E., AND BERNHEIM, A. K.—(1938) *Arch. Path.*, **26**, 971.
LUDFORD, R. J.—(1932) *Sci. Rep. Imp. Cancer Res. Fd.*, **10**, 125.—(1945) *J. nat. Cancer Inst.*, **6**, 89.
Idem AND BARLOW, H.—(1945) *Cancer Res.*, **5**, 257.
MOTTRAM, J. C.—(1928) *Lancet*, ii, 966.
RIES, E.—(1939) *Naturwissenschaften*, **27**, 505.
SHEAR, M. J.—(1944) *J. nat. Cancer Inst.*, **4**, 461.
WOGLOM, W. H.—(1922) *J. Cancer Res.*, **7**, 283.